University of Alberta

Assessment of the Fate of Plant DNA Fragments in the Ruminant Digestive Tract

By

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Abstract

The fate of transgenic DNA from glyphosate-tolerant (Roundup Ready®; R) canola upon ingestion by ruminants was studied in comparison to native canola DNA (i.e., *Rubisco* gene). Whole and cracked seeds, canola meal, and diets containing canola meal, both from R and a near-isogenic parental line, were used in examining the effects of feed processing and exposure to mixed ruminal culture and duodenal fluid (DF). DNA fragments up to 23 Kbp and the entire 1363-bp *cp4 epsps* transgene from RR canola were detectable throughout feed processing. The presence of transgene fragments was determined using conventional and real-time PCR and nine primer pairs amplifying gene fragments of 62 to 527 bp, as well as the whole transgene. No evidence of transfer of transgenic fragments to ruminal microorganisms was found. Ruminal persistence of transgene fragments and of *Rubisco* from R or parental canola were indistinguishable, both associated with plant debris, and inversely related to degree of feed processing. The fate of DNA transported to the duodenum in intact plant cells was studied *in vitro*. Free DNA was rapidly degraded in DF at neutral pH (simulating distal duodenum), suggesting that uptake into tissues via Peyer's patches is unlikely. Transgene was not detected in blood or feces of sheep fed RR canola in forage- or concentrate-based diets, nor was DNA transfer to duodenal microbes detected. Dietary fibre level (i.e., passage rate) did not affect persistence of ingested DNA. In all respects studied, transgenic DNA was indistinguishable from endogenous DNA. Inclusion of data from testing the digestive fate of transgenes present in R canola was suggested to be unwarranted as part of the feed/food safety assessment. Real-time PCR used to quantify transgene fate and persistence revealed a potential correlation with feed degradation. Thus, the technique was used in developing a novel method for estimating dry matter disappearance for individual feed components in a mixed ration. The method was relatively accurate, yielding a correlation of 0.87 between predicted and actual dry matter

disappearances, and for the first time, allowed direct quantification of individual plant digestion in a mixed ration.

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List of Abbreviations

A100	Diet containing 100 % (w/w) alfalfa
A75:C25	Diet containing 75:25 % (w/w) alfalfa:corn
A50:C50	Diet containing 50:50 % (w/w) alfalfa:corn
A25:C75	Diet containing 25:75 % (w/w) alfalfa:corn
A:C	Acetate:Propionate
ADF	Acid detergent fibre
ARMs	Antibiotic resistant markers
bp	base pairs
C	Concentrate-based
C100	Diet containing 100 % (w/w) corn
CCAC	Canadian Council of Animal Care
CFIA	Canadian Food Inspection Agency
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase protein from the CP4 strain of *Agrobacterium tumefaciens*
cp4 epsps	*epsps* transgene from the CP4 strain of *Agrobacterium tumefaciens*
C_T	Cycle threshold
DF	Duodenal fluid
DM	Dry matter
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase protein
epsps	Gene encoding 5-enolpyruvylshikimate-3-phosphate synthase protein
F	Forage-based
FAM	Reporter dye 6-carboxyfluorescein
FAO	Food and Agriculture Organisation
GIT	Gastrointestinal tract
GM	Genetically modified
GMO	Genetically modified organism
GT73	Transgenic canola tolerant to glyphosate
LOD	Limit of detection
LOQ	Limit of quantification
NDF	Neutral detergent fibre
OECD	Organisation for Economic Co-operation and Development
P	Parental

PCS	Parental cracked seed
PD	Parental diet
PCM	Parental meal
PCR	Polymerase chain reaction
PL	Parental leaf
PWS	Parental whole seed
R	Roundup Ready®
RCM	Roundup Ready® canola meal
RCS	Roundup Ready® cracked seed
RD	Roundup Ready® diet
RF	Ruminal fluid
RFC	Readily fermentable carbohydrate
RFLP	Restriction fragment length polymorphism
RL	Roundup Ready® leaf
Rubisco	*ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit* gene
RWS	Roundup Ready® whole seed
TAMRA	Quencher dye 6-carboxytetramethylrodamine
VFA	Volatile fatty acid
WHO	World Health Organisation

Chapter 1 – Introduction

1.1. BACKGROUND INFORMATION

Advances in molecular biology and recombinant DNA techniques have made it possible to engineer plant genomes. Compared with traditional plant breeding methods, such as artificial crossing or hybridization, biotechnology now allows for the introduction of DNA from outside the plant kingdom. Selective inclusion of single or multiple traits can be performed in a precise manner to change the quality of agricultural crops (Gassen and Hammes, 2001). The majority of genetically-modified (GM) crops currently produced have been engineered to enhance agronomic performance by transformation with genes encoding pest resistance or herbicide-tolerance (James, 2004). However, the potential of GM plants is not limited to agronomic enhancement but may also serve as a means of enhancing the nutritional status of feed for livestock or food for humans (Matissek, 1998). In 1996, the first GM crops that act as major feedstuffs for livestock entered the market in North America. These included herbicide-tolerant soybeans and canola and pest-protected corn and cotton. During the nine-year period 1996 to 2004, the global area of GM crops increased over 47 fold. In 2004, GM crops were grown on a total of 81.0 million hectares world wide (James, 2004). Regulations concerning GM plants were established by major international organizations prior to their commercialization. The policy of substantial equivalence was first introduced by the Organisation for Economic Co-operation and Development (OECD, 1993) and was adopted by both the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as the most appropriate method to ensure the safety of GM plants (FAO/WHO, 2000). Substantial equivalence provides a framework for the safety assessment by comparing similarities and differences between a biotechnology-derived plant and an appropriate counterpart such as the parental line the GM plant was derived from. Once certain factors of the plants have been determined to be equivalent, the differences, which may only relate to the novel transgenic trait, become the focus of the safety assessment. Detailed information about the feeding qualities of GM plants for livestock and their nutritional evaluation have been reviewed previously (Flachowsky et al., 2005; Aumaitre et al., 2002; Flachowsky and Aulrich, 2001).

Throughout the world, there is still controversy at public and scientific levels about the benefits and risks regarding GM crops entering the food- and feed-chain as well as present regulations in place (Finucane, 2002; Shelton et al., 2002). Between 1996 and 1999, Europeans

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became increasingly opposed to GM foods (Gaskell et al. 2000). A more recent study showed that European consumers place significantly greater value on beef from cattle fed conventional corn grain as compared to those fed genetically-modified corn (Lusk et al., 2003).

In particular, there has been interest in the stability and presence of recombinant DNA or proteins expressed by that DNA entering the food chain. Given that livestock consume large amounts of plant material and that high-protein feeds are among the most common GM crops, there is considerable opportunity for livestock to consume significant quantities of recombinant DNA and protein. The objective of this chapter is to review the global status of GM crops and current literature on the fate of transgenic DNA consumed by livestock.

1.2. GLOBAL STATUS OF GM CROPS

1.2.1. Area of GM crops grown worldwide

In 2004, for the ninth consecutive year, the global area of planted GM crops increased (James, 2004). Starting from 1.7 million hectares in 1996, the use of GM crops has grown by more than 47 times to 81.0 million hectares. This accounted for 5% of the 1.5 billion hectares of all cultivable crop land. The number of countries that cultivated GM crops in 2004 totaled 17 and included 11 developing and six industrialized countries. Fourteen of these countries grew GM crops on 50 000 or more hectares. From 2003 to 2004, there was a 20% increase in land dedicated to GM crops, and for the first time since their commercialization, growth of the total GM crop area from the previous year was greater in developing countries than industrial countries. This trend will likely continue as more farmers in those countries adopt GM crops. Developing countries accounted for 34% of GM crop usage in 2004. However, as new traits are introduced, growth will also likely continue in industrialized nations.

1.2.2. GM traits and crops

The majority of GM plants grown to date have been engineered to improve agronomic performance. While the number of traits of GM plants that have undergone some form of regulatory review is greater than 10 (AgBios Database, 2005), the principal traits of the most popular plants in 2004 were those for herbicide-tolerance (58.6 million hectares), insect-resistance (15.6 million hectares), or a combination of both (6.8 million hectares) (James, 2004). The major GM crops grown in 2004 were: soybean, planted on 48.4 million hectares (60% of the

global GM area); maize at 19.3 million hectares (23% of global GM area); cotton at 9.0 million hectares (11% of planted GM crops); and canola at 4.3 million hectares (6% of the global GM area). Cultivation of GM varieties of each of these crops increased from the year before.

The most popular GM crops grown worldwide are also extensively used in diets fed to livestock. In Canada, beef cattle alone consumed 139, 100 tonnes of canola meal, 207, 900 tonnes of soybean meal, and 733, 300 tonnes of corn grain throughout 2001 (Statistics Canada, 2003). Canada has seen a rapid adoption of GM canola, which accounted for 65% of all canola grown in Canada in 2002 (James, 2002). The United States on the other hand, grew the largest amount of GM soybean that same year, with 79% of its total production being of GM origin. It therefore seems apparent that livestock animals in North America consume GM plants or plant products on a regular basis and this is likely to be the case for other countries growing GM crops or importing them for use as feed.

1.3. GM FEED AND LIVESTOCK

1.3.1. Rationales for animal studies

Although regulations with regard to GM plants have been developed primarily from the perspective of human consumption of GM food, it is generally assumed that these same criteria are suitable for a risk assessment of the consumption of GM feed by livestock. The European Commission has a combined safety approach that requires an assessment of risks for humans, animals, and the environment before approval of importation or cultivation of a novel crop (EC, 2001). In contrast, Canada has specific legislation relating to the safety assessment of novel GM feeds in which all livestock feeds derived from plants with novel traits are subject to mandatory review for safety and efficacy by the Canadian Food Inspection Agency under the Feeds Act and Regulations (CFIA, 1995).

An integral part of the safety evaluation of GM plants is to test for substantial equivalence. Substantial equivalence does not constitue a nutritional safety assessment of a GM plant but rather provides a starting point for the overall assessment (FAO/WHO, 2000). The aim of such a test is to determine whether a transgenic plant is substantially equivalent to its conventional counterpart at a chemical and nutritional level. While there are no formally defined parameters to be measured, minimal analyses performed should determine whether the major nutritional components (i.e., lipids, carbohydrates, proteins, vitamins, minerals, trace elements) and known antinutrients and toxins of transgenic plants are equivalent to conventional varieties

that have a history of safe use. For livestock nutrition, important measurements include crude protein, fat (ether extract), fiber, starch, amino acids, fatty acids, ash and sugar (Aumaitre et al., 2002). Some of these factors are not only capable of affecting animal health and performance, but might alter the composition and quality of animal products provided to the consumer. For example, certain fats present in feeds can affect the composition of fat in animal tissues (Aumaitre et al., 2002). Guidelines for second generation GM crops that are not substantially equivalent have been proposed (Flachowsky and Bohme, 2005).

Animal experiments are important and provide valuable information regarding the safety of a GM plant for both livestock and human consumption. Adverse effects of a plant in livestock should be a warning for human use. In addition, economic concerns regarding animal performance are also addressed. To date, there have been no studies showing adverse effects in any of these areas when the currently registered GM plants or products derived from them have been used as feeds (Flachowsky et al., 2005). It appears that the policy of substantial equivalence also results in nutritional equivalence. Still, there has been significant research regarding the fate of recombinant molecules within GM crops after consumption by livestock. Information on most of the plants listed in the below animal studies and their novel traits have been described by AGBIOS, 2005.

1.4. THE FATE OF TRANSGENIC DNA

1.4.1. Rationales for investigating the fate of transgenes

Concerns regarding transgenic DNA are based on indirect consequences resulting from possible transformation events and the physical presence of transgenic DNA in animal products. The fate of transgenes has been studied to a greater extent than that of proteins because of more issues concerning human health. It has been suggested that the CaMV promoter, which is a regulatory sequence common to most registered GM plants, could cause cancer through over-expression of oncogenes, should the promoter be integrated into human cells through recombinant events after absorption (Ho et al., 1999). However, the cauliflower mosaic virus is ubiquitous and its promoter has been detected in food samples not containing transgenic DNA from GM plants (Wolf et al., 2000). Both FAO and WHO have stated that there is no direct health risk to consumers ingesting transgenic DNA because the DNA from all organisms is structurally similar (WHO, 1991). However, like proteins, the presence of transgenes in animal products could also affect commodity sales. Another concern regarding transgenic plant DNA

that has received attention is the possible transfer of antibiotic resistant markers (ARMs) to bacteria. ARMs in the currently registered GM plants are unlikely to result in the development of resistance to the therapeutic antibiotics presently used in animal and human health (FAO/WHO, 2000). The antibiotics used as markers are either rarely used in human medicine or widespread resistance is already prevalent in nature. For the stable transfer of plant DNA into a microbial or mammalian cell to occur, FAO/WHO (2000) have proposed that all of the following events would have to occur:

- the relevant gene(s) in the plant would have to be released, probably as linear fragments
- the gene(s) would have to survive nucleases in the plant and in the gastrointestinal tract
- the gene(s) would have to compete for uptake with dietary DNA
- the recipient bacteria or mammalian cells would have to be competent for transformation and the gene(s) would have to survive their restriction enzymes
- the gene(s) would have to be inserted into the host DNA by rare repair or recombination events

It is important to note that the gene(s) would have to survive any feed processing events prior to intake by livestock as well. And aside from competing with other plant DNA, the gene(s) would be heavily diluted in microbial DNA and would have to compete with that DNA for uptake into a cell, especially for animals that have sufficient digestion prior to the abomasum, such as ruminants and poultry. Prior to the marketing of GM plants, there was little interest in the fate of plant DNA after consumption by animals. As a result, the knowledge about the fate of ingested feed DNA was limited. In recent years, studies have added significant insight into the fate of plant genes after consumption.

1.4.2. Transgene intake by livestock

The amount of transgenic DNA ingested depends on the concentration of transgene in feed as well as feed intake. The quantity of DNA in most crops is less than 0.02% (w/w) on a DM basis (Beever and Kemp, 2000). As a percentage of total DNA, the transgene concentration is unlikely to change for an individual event because nuclear gene insertions are generally static. However, as a percentage of DM intake, the concentration of transgenic DNA can vary due to environmental conditions that change the overall biomass of the plant, such as lignification with

aging. Different parts of a plant will have variable transgenic DNA content as well, which is dependent on the quantity of cells per gram of DM. In addition, DM transgene content can differ significantly between events of the same crop variety because the genome size of crops are not constant values and can show variation of up to 25% (Van den Eede et al., 2002). Beever and Phipps (2001) estimated that a dairy cow consuming a 24 kg DM/d of a diet containing 40% transgenic maize silage and 20% maize grain, would have an intake of 57 g/day of total plant DNA. Of that, 54 μg would be recombinant DNA and account for only 0.000094% of the total DNA intake. The actual total of transgenic DNA intake may actually be lower, considering that ensiling GM plants quickly leads to degradation of large plant DNA fragments (Hupfer et al., 1999). Other processes too, such as heat treatment, will also lower the intake of intact transgenes.

1.4.3. Ruminants

The first studies investigating the fate of plant DNA in ruminants took place more than three decades ago and showed that most plant DNA, whether fed as a free form or as whole plant, is quickly degraded to oligonucleotides, nucleosides and bases throughout the digestive tract (Smith and McAllan, 1971; McAllan and Smith, 1973; McAllan, 1980; Razzaque and Topps, 1981). These studies focused on total nucleic acids and did not attempt to describe differential plant gene digestion partly because this was not of concern at the time and partly because the molecular techniques to do so were yet to be developed. Subsequent to the introduction of GM plants, numerous reports have attempted to describe the persistence of transgenes.

Deoxyribonuclease activity has been shown to be present in rumen fluid (Flint and Thomson, 1990; Duggan et al., 2000; Ruiz et al., 2000) and intestinal fluid (Alexander et al., 2004). This likely explains why most plant DNA genes, at least those present at low copy numbers, have been shown to be mainly associated with feed residue. Phipps et al. (2003) analyzed gene stability throughout the digestive tracts of dairy cows fed 18.5% insect-resistant corn (event MON810) and 13.0% herbicide-tolerant soybean meal (event GT 40-3-2). In the liquid phases of both ruminal and duodenal fluids, only a 167 bp sequence of the high copy chloroplast *rubisco* gene was detected. In contrast none of the low copy amplicons from the endogenous soybean *lectin* (240 bp) and recombinant (171 bp) genes or the endogenous maize *HMP* (209) and recombinant (203 bp) genes could be detected. All of the fragments were amplifiable from the solid phases of each digestive fluid. In the feces, only the *rubisco* gene was detected. Similar results were reported for the 1363 bp *cp4 epsps* transgene found in herbicide-tolerant canola (event Gt73) when canola substrates were incubated in ruminal batch cultures

(Alexander et al., 2002). The same was also true for smaller fragments of the transgenic construct in Gt73 canola substrates, ranging in size between 300 and 527 bp, when incubated in ruminal batch cultures (Sharma et al., 2004). A 62 bp sequence of the transgenic construct however has been detected in the aqueous phase of intestinal fluid *in vitro* but the copy number of this amplicon only reached a maximum number of 1600 copies when digestion was at its greatest (Alexander et al., 2004). The small amplicon size likely affected these results, as the entire 1363 bp transgene was not detected in the liquid phase.

Einspanier et al., 2004 used real time PCR to quantify transgenic and endogenous genes throughout the digestive tract of cattle fed diets containing 88.5% (w/w) Bt176 maize silage. After ensiling, the quantity of each gene decreased to less than 3% of the starting quantity. Surprisingly, the amounts of both transgene and endogenous gene seemed to increase after passage from the rumen to the abomasum, before decreasing dramatically to unquantifiable levels in the jejunum and colon. It should be noted that the quantity of each gene was expressed per 90 ng of total DNA. Therefore, it is likely that the plant genes were diluted with microbial DNA to a greater extent in the rumen than the abomasum. Both the transgene and endogenous gene followed similar trends throughout the gastrointestinal tract (GIT).

Should plant DNA fragments be absorbed, the likely place for such an event would be the intestine, and more specifically, the Peyers patches of the distal ileum or proximal large intestine (Schubbert et al., 1997). Because of the highly unstable nature of DNA in the ruminant digestive tract, it is probable that DNA released in the rumen, at least for low copy genes, does not persist to the proximal small intestine. Therefore, digestion of plant residue in the ileum may be necessary for plant genes to have a chance of crossing the intestinal barrier. There is evidence that digestion does occur in the ileum (Alexander et al., 2004; Erfle et al., 1982) and that plant DNA is released into the aqueous intestinal phase. The first study to probe for plant DNA in ruminant products showed that transgenic DNA was not detectable in the muscle, liver, spleen, kidney, and blood lymphocytes of cattle fed ad libitum Bt 176 silage (Einspanier, 2001). However, given the reduction in transgene concentration during ensiling, these results are not surprising (Einspanier et al., 2004). A 199 bp chloroplast sequence of DNA could be detected in the blood lymphocytes however. Similarly, a *rubisco* gene fragment was detected in the blood of cattle fed GM corn and soybean meal, but transgenic sequences were never detected (Phipps et al., 2003). The same occurred for calves being fed rations containing 43.3% (w/w) Bt11 insect-resistant corn (Chowdhury et al., 2004). A 231 bp fragment of the *rubisco* gene was detected in the liver, spleen, kidney, mesenteric lymph nodes, and longissimus muscle samples. However, the *Cry1Ab* transgene tested negative in all of the tissue samples. Nemeth et al. (2004) were also

able to detect a 173 bp sequence of the *rubsico* gene in the beef brisket muscle of cattle fed 75% (w/w) dry rolled corn and 15% (w/w) corn silage (event MON810) for 5% of the samples, whereas transgenic DNA was not detected. The same authors also tested for plant DNA in milk from dairy cattle fed 20% corn plus 60% corn silage of the same MON810 event. A 173 bp sequence of the *rubisco* gene was amplifiable in 86% of the samples, and indeterminate in the other 14%. A larger 500 bp sequence of the same gene could be detected in 79% of the samples, while the remaining 21% were indeterminate.

Milk from dairy cows fed two varieties of insect-resistant cotton seed (*Cry1Ac* gene and C*ry1Ac* plus *Cry2Ab* genes) and two varieties lacking the C*ry1Ac* transgene at 11% of DMI have been analyzed (Castillo et al., 2004). Neither a 215 bp segment of the transgene or a 400 bp segment from the endogenous *acyl carrier protein* gene were amplifiable from any milk sample. Other studies attempting to detect transgenes and low copy endogenous genes in milk from animals fed GM cotton (Jennings et al., 2003a), corn (Phipps et al., 2003; Jennings et al., 2003a; Yonemochi et al., 2003), soybean (Phipps et al., 2002) or non-GM corn and soybean (Poms et al., 2003) have been unsuccessful, suggesting that while absorption of plant DNA is possible throughout the ruminant digestive tract, passage of foreign plant DNA into milk is unlikely.

1.4.4. Swine

Chowdhury et al. (2003a) analyzed transgenic and endogenous DNA fragments by PCR throughout the intestinal tract of pigs fed diets containing 70% (w/w) insect-resistant corn. Primer sets detected two fragments of the endogenous *zein* gene (242 bp and 329 bp) and two fragments of the transgenic construct that encoded the *Cry9C* gene (103 bp and 170 bp). None of the plant sequences were detected in the duodenal contents. However, this was likely a result of feed passage to the large intestine, as sampling of digestive contents took place the day after the last feeding. Both endogenous and transgenic primer sets detected their respective DNA fragments in cecal and rectal contents of the pigs (range of 25-50% of the samples were positive), indicating that plant DNA is detectable towards the end of the swine digestive tract when such a diet is consumed. Another study detected three separate endogenous genes and two transgenic fragments of varying size from digestive contents of the stomach, duodenum, ileum, cecum, and rectum of 10 pigs fed diets containing 60% (w/w) insect-resistant corn (Chowdhury et al. 2003b). All of the endogenous fragments, which included sequences from the *Rubisco* (1028 bp), *invertase* (226 bp), and *zein* (242 bp) genes were detected in 30-100% of the samples, depending of the origin of the contents and primer set. The relatively large amplicon size of the *Rubisco*

gene indicates that even substantially sizeable fragments of DNA survive the digestive process in swine. However, the authors noted that corn kernels were visible within gastrointestinal contents. It is therefore possible that DNA detected towards the end of the digestive tract was protected within undigested corn residue that was inaccessible to DNA-degrading enzymes. Unlike ruminant studies, the relative stability of free DNA in swine digestive contents has not been reported. The two transgenic fragments (110 bp and 437 bp) were located within a construct containing the *Cry1Ab* gene. Each sequence was detected to the same extent in the stomach (100%), however the larger fragment was detected less frequently than the small fragment in duodenal (100 *vs.* 60%), ileal (100 *vs.* 60%), cecal (100 *vs.* 60%) and rectal contents (60 *vs.* 20%). It cannot necessarily be stated that the smaller fragment was more persistent than the larger one because the limit of detection (LOD), which was not reported, could have resulted in these differences in sensitivity of detection. Generally, smaller amplicons result in greater PCR efficiency. In theory, if undigested plants residue and therefore undigested plant cells are present in a digestive sample, then all parts of the genome should be amplifiable. Whether plant DNA persists in a free form or is feed-associated throughout the swine digestive tract remains to be investigated.

Klotz and colleagues (2002) attempted to describe the time-dependent persistence of plant DNA in the upper part of the GIT of pigs. The animals were switched over from a non-transgenic diet and fed 1 kg of feed containing 50% insect-resistant corn (event 176), after which they were slaughtered at sequential times up to 12 h. A 199 bp sequence of plant chloroplast DNA was amplifiable from contents of the stomach, duodenum, jejunum, and ileum for 12 h after feeding, although the intensity of each PCR product eventually diminished over time. In contrast, transgenic sequences of the *Cry1Ab* gene (211 and 251 bp) were not detected from any digestive sample at any time point. The differences are likely due to the number of copies of each gene per genome. Chloroplast DNA genes can be present between 500-50000 copies (Bendich, 1987) compared to the single insert of most transgenes in nuclear DNA. In addition, neither the endogenous or transgenic DNA sequences could be detected in blood or lymph nodes from the animals at any time point. These results are in contrast to those reported by Reuter and Aulrich (2003) who also performed gene persistence studies using Bt176 corn. Animals in this study were fed 2.6 kg of a ground diet (1 mm particle size) containing 70% (w/w) corn throughout the fattening phase. A 211 bp fragment of DNA amplified from the *Cry1Ab* gene was detected in the stomach up to 24 h, the duodenum, jejunum and ileum up to 48 h, the cecum up to 12 h, colon up to 24 h, and rectum up to 48 h after feeding the diet. A 140 bp fragment from chloroplast DNA was detected in every type of sample taken from the GIT, even 72 h after feeding. While it might

be expected that DNA digestibility would increase with the degree of grinding of the feed, as is the case in the study by Reuter and Aulrich (2003), the amount of transgenic DNA ingested will also affect the likelihood of gene persistence within the digestive tract. This may explain the differences in results between the above two papers. Klotz et al. (2002) fed the pigs 0.5 kg of insect-resistant corn whereas the animals in the study by Reuter and Aulrich (2003) were fed 1.82 kg of corn per day.

The above two studies also attempted to detect plant DNA in animal tissues. Reuter and Aulrich (2003) were able to detect the 140 bp fragment for chloroplast DNA in blood, liver, lymphatic glands, spleen, kidney, *musculus gluteus maximus*, *musculus*, *longissimus dorsi*, *musculus trapezius* and ovary samples in 16.7, 54, 16.7, 12.5, 27, 33.3, 54.2, 22.9, and 62.5% of the samples tested, respectively. Again emphasizing the importance of transgene copy number in digesta detection, the transgenic DNA from the single copy *Cry1Ab* gene was never detected in any sample. The relatively high detection rate of the chloroplast DNA in the ovaries was suggested to result from high blood flow to that organ. Klotz et al. (2002) were also unable to detect transgenic DNA in muscle, liver, spleen, lymph nodes and blood from pigs fed diets containing 20-25% (w/w) transgenic corn. In addition, and again in contrast with Reuter and Aulrich (2003), chloroplast DNA was also undetectable in any of the mentioned tissues. These differences between and within each study likely highlight the significance of the number of genes ingested, or perhaps method sensitivity. The diet fed by Reuter and Aulrich (2003) was finely ground thus particle size was unlikely to be a factor in gene persistence in this study. The animals in the study by Klotz et al. (2002) consumed fewer copies of transgenic and endogenous maize genes, as mentioned above. With regard to the differences in detection of plant-specific chloroplast DNA, it is possible that the amount of each chloroplast sequence differed at a genome level and thus intake within each experiment could have been substantially different, as the copy number of the chloroplast targets were not reported. Additionally, the intake of the transgene compared to the chloroplast gene is low which may have resulted in differential persistence and detection of the two genes.

Another factor that will directly affect gene persistence throughout the digestive tract and therefore indirectly affect the chance of passage across the GIT epithelia is digestibility of the test plant. Higher digestible feedstuffs, such as soybean meal, are likely to have their DNA degraded more rapidly, decreasing the chance for absorption. Jennings et al. (2003a) attempted to detect a low copy endogenous and recombinant gene in longissimus muscle samples from pigs fed 24-14% (w/w; grower to finisher phases, respectively) herbicide-tolerant or conventional soybean meal. The study combined PCR followed by Southern hybridization to create highly sensitive

test assays for sequences of the intrinsic *lectin* gene (198 bp, LOD = one diploid genome equivalent) and recombinant *cp4 epsps* gene (272 bp, LOD = one diploid genome equivalent). None of the samples tested positive for either gene.

The presence of plant DNA in 118 samples from the *longissimus* muscles of pigs fed approximately 85% (w/w) insect-resistant corn (event MON810) or an isogenic control has also been explored (Nemeth et al., 2004). These authors used highly sensitive primers, as indicted by low LODs, that amplified two fragment lengths from a chloroplast *rubisco* gene (173 bp, LOD = 0.02 genome equivalents; 500 bp, LOD = 0.08 genome equivalents) and a fragment from the *p35S* gene (123 bp, LOD = 5 genome equivalents) that is the promoter of the transgenic construct. If any of the muscle samples tested positive for the p35S gene, then analysis for MON810 construct-specific sequence (149 bp, LOD = genome equivalents) was carried out. For the 173 bp *rubisco* sequence, 53% of the samples tested positive (both duplicates positive), 43% were negative (both duplicates negative) and 4% were indeterminate (duplicate samples were both positive and negative). The 500 bp *rubisco* fragment proved to be positive in 43% of the samples, negative in 43% and indeterminate in 14%. One tissue sample out of the total 118 tested positive for the *p35S* sequence. To confirm the results, the analysis was repeated with new tissue subsamples, which again were positive. However, when tested with the MON810 primer set, the result was indeterminate. This suggested that the number of transgene copies was below the LOD. Statistical analysis showed the positive result to lack significance. However, the actual occurrence of such an event is likely to happen at such a low rate that for it to be significant, an extremely large sample population would be required. The importance of this result is that transgenic DNA acts similarly to endogenous DNA, which when present in enough quantity, might cross the gastrointestinal barrier. Additionally, despite detection of the transgene in pork tissue, the performance study that these samples were analyzed from, showed no effect on growth performance or pork quality between pigs fed diets containing transgenic or conventional corn (Weber et al., 2000). Thus neither transformation of the corn nor the transgene itself adversely affected animal health.

1.4.5. Poultry

Chambers et al. (2002) investigated the fate of plant DNA throughout the digestive tract of chickens fed insect-resistant maize (event 176) present at 80% of the diet. PCR-restriction fragment length polymorphism (RFLP) analysis indicated that the *beta lactamase* (*bla*) gene, present as part of the transgenic construct found in 176 maize, could be detected in the crops of

each bird tested (N=5) and in the stomach of two of the birds. Results for the transgenic DNA were negative in the small intestine, large intestine, cecum, and rectum. Detection of the maize mitochondrial gene, *nad5*, showed similar detection, being present in the crop and stomach of all birds. Mitochondrial genes are generally present at a higher copy number, probably resulting in better detection from digesta contents of these two segments. The *nad5* gene was also not detected in any of the other digestive contents. A study testing diets containing 60% (w/w) of the same insect-resistant corn supported the findings of Philip and colleagues (Aeschbacher et al., 2005). The transgenic *bla* (479 bp) could be detected in the crop of broilers by PCR and not in the gizzard, small intestine, cecum, and excreta. The endogenous *invertase* gene was also limited mostly to the upper part of the digestive tract, being detected in the crop, gizzard, and to a lesser extent, the small intestine. Contradicting the above two studies was an experiment that detected DNA throughout the GIT of poultry fed diets with the same Bt176 maize at an inclusion rate of 74% (w/w; Tony et al., 2003). Using real time PCR, the authors showed that sequences of the maize-specific *hmg* gene (79 bp) and the transgenic *Cry1Ab* gene (129 bp) were detected in the crop, proventriculus, gizzard, duodenum, jejunum, ileum and cecum and rectum. Although quantitative data was not presented, the threshold cycle was reported for each of the digesta contents up to 24 h after starvation. The threshold cycle is inversely proportional to the amplicon quantity in a sample. The *hmg* gene showed a similar detection pattern between birds fed transgenic corn and an isogenic control and was also similar to that of the *Cry1Ab* gene in birds fed transgenic corn. For the most part, the threshold cycle for both amplicons generally increased with increasing time of starvation. This likely reflects a combination of passage of the genes throughout the GIT and degradation. The authors suggested that maize DNA can persist throughout the GIT without much degradation and is excreted in the fecal matter. This may be misleading however, when the threshold values are considered. In some cases, the threshold values increase from about 25 at 0 h to approximately 38 at 24 h. Theoretically, each increase or decrease in threshold value represents a two-fold change in gene copy, assuming an efficient PCR. Therefore, a threshold difference of 13 units reflects a change in concentration of 4096 times. This implies significant degradation or passage over time. However, the differences in persistence between the studies may also reflect smaller PCR products in the latter experiment.

Using conventional PCR, Tony and colleagues (2003) were unable to detect both a 211 bp sequence of transgenic DNA and 226 bp sequence of the endogenous *invertase* genes in blood, pectoral, thigh, liver, heart, spleen, kidney, bursa, or thymus tissues from broilers fed diets containing 73.6% (w/w) Bt176 corn. Like the trangene, the *invertase* gene is a low copy gene and is present at one copy per plant genome (Hernandez et al., 2004). However, a 199 bp

sequence of high copy chloroplast DNA was detected in all these tissues except the heart, bursa, and kidney, up to 4 h after starvation. Aeschbacher et al. (2005) reported similar results for transgenic DNA detection for birds fed diets containing 60% (w/w) Bt176 corn. The transgenic *bla* gene (479 bp fragment), was not identified in the liver, spleen, muscle, blood, and eggs of hens or broilers but was faintly identifiable in the crop of broilers. The authors however were able to detect the same low copy 226 bp *invertase* sequence that Tony et al. (2003) had used, in the liver, spleen, muscle, blood, crop, gizzard, and small intestine of broilers but not in the cecum or excreta. Because all DNA behaves similarly, it would be expected that the absorption of one low copy gene fragment should signify the absorption of other low copy gene fragments, including those from the transgenic *bla* gene. However, fragment absorption may be size-dependent and the authors did note that the majority of DNA recovered from the digestive tracts of the birds were less than 180 bp, smaller than the amplicon size of the *bla* primer set.

Klotz et al. (2002) also attempted to detect plant DNA in poultry tissues and have provided evidence of a size-dependent limitation to fragment absorption. Commercial market samples of breast, leg, stomach, and wing tissues from turkeys and chickens were tested for fragments of endogenous maize genes, including a 532 bp fragment of chloroplast DNA, a 199 bp fragment of chloroplast DNA, and a 277 bp fragment of the *zein* gene. None of the samples were positive for the 532 bp chloroplast sequence however, the 277 bp fragment was detected in all of the samples. Although the LOD was not reported for the primer sets, it probable that shorter DNA fragments more readily cross the intestinal barrier. The *zein* gene, which is a low copy gene present at roughly two copies in the haploid maize genome (Hernandez et al., 2004) was also faintly detected in the samples. The authors did not test for transgenic DNA but from this study and that by Tony et al. (2003), it appears that short fragments of low copy plant genes have the potential to be absorbed throughout the avian digestive tract and present in adult tissues. In contrast, the DNA fragments were not detected in the embryos of eggs. Einspanier et al., 2001, were also unable to detect the same 199 bp chloroplast fragment in the eggs from chickens fed diets containing 50% (w/w) Bt 176 maize. Taken together with the above study, it seems that plant DNA is less likely to cross into the eggs of chickens. The fragment was amplified in muscle, liver, spleen, and kidney tissues. However, a 189 bp segment of transgenic DNA was not detectable in any of the samples.

Two studies have also tested for transgenic DNA in tissues of chickens fed diets containing approximately 60% of insect-resistant MON810 corn (Jennings et al., 2003c; Nemeth et al., 2004). Again using a PCR-Southern hybridization method, Jennings and colleagues (2003b) were able to detect one copy of the diploid genome for both the 213 bp endogenous *sh2*

and 211 bp transgenic *Cry1Ab* primer sets. Both genes are present at low copy numbers but despite adding 1 ug of extracted muscle DNA to the PCR reactions, neither gene sequence was detected in any of the samples. Nemeth et al. (2004) were unable to detect the 149 bp segment of the transgenic construct in breast muscle, however a 173 bp sequence of the high copy chloroplast *rubisco* gene was positive in 15% of the samples, negative in 75% of the samples, and indeterminate in 10% of the samples, again emphasizing the importance of gene copy number.

1.5. CONCLUSION

Agriculture has evolved to produce and select plants with more desirable traits. Modern recombinant DNA technology has allowed for the precise and timely introduction of DNA from any source into a plant. The global area of GM crops increased to 81.0 million hectares in 2004. To ensure the safety of GM plants as animal feed, regulatory bodies have adopted the policy of substantial equivalence. Though this does not prove nutritional equivalence, to date, there have been no adverse effects in animals consuming commercialized GM crops that have been approved on the basis of substantial equivalence. Studies undertaken to address concerns that transgenic DNA may enter the market by means of animal products have shown that recombinant materials in transgenic feed are unlikely to be incorporated into animal products at a significant level. Absorption of plant DNA across the intestinal barriers of livestock does seem to be a normal occurrence when DNA fragments are present at high concentrations. Absorption of DNA does not appear to have adverse effects on livestock, whether the DNA is transgenic or endogenous. Given the popularity of GM crops, which is expected to increase more over the next few years, GM plants in the food-chain should continually be monitored. Rigorous testing procedures for novel crops should remain in place, especially when traits are introduced that affect nutritional components. In these instances, nutritional equivalence should be determined by performing animal trials, in addition to substantial equivalence tests. However, the fate of recombinant molecules in the currently registered GM plants does not need to be included in feed safety assessments.

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Chapter 2 – Impact of Feed Processing and Mixed Ruminal Culture on the Fate of Recombinant *epsp* Synthase and Endogenous Canola Plant DNA

2.1. INTRODUCTION

The increasing use of genetically-modified (GM) crops has raised numerous questions regarding the fate of recombinant plant DNA in animals, humans as well as microbial populations inhabiting these systems. In particular, there are major concerns regarding the uptake and persistence of foreign DNA in animal and bacterial cells (Gasson 2000). Few studies to date have investigated the persistence and survival of DNA throughout the animal digestive tract. Studies involving mice have shown that ingested foreign DNA can persist long enough to cross the intestinal epithelium, and reach leukocytes, spleen and liver cells of the host as well as various fetal organs Schubbert et al. 1997, 1998). Upon feeding soyabean leaves to mice, plant-specific ribulose-1,5-bisphosphate carboxylase/oxygenase gene was detected in the liver and spleen (Hohlweg and Doerfler 2001). Similar findings have been reported in a study involving chickens and cattle fed GM-based diets (Einspanier et al. 2001), where small fragments of endogenous plant DNA were identified in the muscle, liver, spleen and kidney of chickens. In ruminants, detection of the same plant gene fragments was limited to blood lymphocytes and to a lesser extent, the milk in cattle. These differences in gene detection from cattle and chickens may have been due to a greater extent of plant digestion prior to plant material reaching the intestinal tract in the former. Recombinant DNA was not detected in any of the tissues of either species.

Ruminants consume vast amounts of plant DNA each day and host a diverse and concentrated microbial ecosystem throughout their digestive tract. Despite this, little is known about the fate of plant DNA upon ingestion by these animals. The acquisition of plant DNA by microbes would most likely occur by transformation. There are bacterial species known to be capable of natural transformation (Neilsen et al. 1998), including those present in rumen (Mercer et al. 1999). Fungal species too have been shown to be capable of transformation (Fincham 1989). Most studies on DNA stability and transformation however, have been conducted under ideal *in vitro* conditions and thus have limited practical applications. In order for natural transformation to occur, free DNA has to be available for uptake. It has been observed in ruminants that naked DNA is degraded very rapidly, thus imposing an initial barrier to transformation with plant DNA (McAllan and Smith 1973; Duggan et al. 2000). Though DNA has been reported to lose the capacity to transform bacteria within 1 minute of incubation in rumen fluid (Duggan et al. 2000),

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transfer of genes across species within the rumen does seem possible (Garcia-Vallve et al. 2000).

Roundup Ready® canola is glyphosate tolerant due to expression of recombinant DNA encoding 5-enolpyruvylshikimate-3-phosphate synthase (*cp4 epsps* or *cp4 epsp* synthase). The purpose of this study was two-fold: firstly, to monitor plant DNA throughout feed processing of parental and Roundup Ready® canola in order to determine the size of plant DNA consumed by ruminants when fed processed diets. The second part of our study addressed the persistence of transgenic (*epsp* synthase) and endogenous canola plant DNA (Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit or Rbc) during feed digestion by mixed ruminal microbial populations.

2.2. MATERIALS AND METHODS

2.2.1. Canola seed, meals and diets

Parental and Roundup Ready® canola whole seeds were supplied by Monsanto Company (St. Louis, MO). The respective canola meals were prepared at Texas Engineering Experiment Station, Texas A & M University (TX, USA). Briefly, the canola seeds were conditioned (average discharge temperature 74.4°C) and flaked to a thickness of 0.254-0.381 mm. The flakes were then cooked (discharge temperature 96.1-101.7°C), dried, and extracted in hexane heated to 54.4°C (1 part solvent:1 part solid). The meal was recovered at 93.9°C.

The diets from the respective meals were prepared at the Lethbridge Research Centre (Lethbridge, AB, Canada) and contained 6.5% (w/w; as fed) of each of the canola meals (Parental or Roundup Ready®) on a whole percentage basis. For preparation of the diets, canola meal was mixed with the other constituents of the diet and pelleted at 100°C.

2.2.2. Canola leaf

Leaf samples were obtained from canola plants grown in a phytotron facility at the Lethbridge Research Centre under standard conditions and were used as positive or negative controls (as indicated).

2.2.3. Batch culture substrates

For batch culture experiments, the substrates tested were parental (P) and Roundup

Ready® (R) of each of the following: whole canola seeds (PWS and RWS), cracked canola seeds (PCS and RCS), meals (PCM and RCM), and diets (PD and RD). Whole canola seeds were cracked using a Wiley mill. Meals and diets were ground sufficiently to pass through a 1-mm screen.

2.2.4. Batch culture fermentation

To prevent possible background and contamination from *cp4 epsps* canola or soybean containing diets, a rumen fistulated Jersey steer maintained on alfalfa hay was used in this study. The rumen contents were collected, via a cannula, two hours after the animal was let out to graze. Approximately 1.5 L rumen fluid was collected by straining digesta through four layers of cheesecloth into a flask flushed previously with CO_2. Solid digesta was sealed in a bag and both fractions were taken back to the laboratory for processing, under anaerobic conditions.

Inoculum was prepared by blending 1.5 L rumen fluid with 375 g of solid digesta for three 45-s pulses. The homogenate thus obtained was passed through four layers of cheesecloth and the strained fluid was added to two volumes of buffer pre-warmed to 39°C as previously described (Menke et al. 1979) to obtain the rumen fluid inoculum. The rumen fluid inoculum (20 mL) was dispensed into triplicate 35 mL serum vials containing 250 mg of each of the substrates (PWS, RWS, PCS, RCS, PCM, RCM, PD, or RD). The vials were sealed and incubated at 39°C on a rotary shaker for 0h, 2h, 4h, 8h, 12h, 24h, or 48h. The 0h vials were processed immediately after the addition of inoculum. For the remaining incubations, triplicate vials were removed from the shaker at the indicated time points and placed on ice prior to processing. The batch culture fermentations were processed step-wise to obtain feed particle associated bacteria, fluid associated bacteria and supernatants as previously standardized in our lab (Hristov et al. 2001). Briefly, the complete contents of each vial were transferred into a 50 mL Falcon tube, and centrifuged at 500 × *g* (10 min; 4°C). The supernatant (supernatant A) was aliquoted for Volatile fatty acid (VFA) measurements and the remaining pellet (pellet A), containing plant debris and feed-particle associated bacteria (Hristov et al. 2001), was used for DNA isolation. A sub-sample (2 mL) of the supernatant A was centrifuged at 10 000 × *g* (4 min, room temperature) and the resulting supernatant (supernatant B) which contained free DNA was transferred to a fresh 2 mL tube and used for DNA isolation. The remaining pellet (pellet B), containing fluid-associated bacteria (Hristov et al. 2001), was also used for DNA isolation. VFA concentrations in the supernatant from the first centrifugation step (500 × *g*) were determined by gas chromatography as described previously (McAllister et al. 1998).

2.2.5. DNA isolation

DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, Canada) was used for isolating DNA from canola plant leaves (parental and Roundup Ready®) which were used as controls.

DNA isolation from batch culture fermentations of seeds, meals and diets was accomplished by using a modified CTAB extraction from a previously published procedure (Ausubel et al. 1995). Briefly, 1 g of seed, meal or diet was ground in liquid nitrogen using a pestle and mortar. The ground material was added to 4 mL of 2-mercaptoethanol/CTAB solution pre-warmed to 65 °C in a 50 mL centrifuge tube, capped, and incubated for 1 h. An equal volume (4 mL) of chloroform/isoamyl alcohol (24:1) was added and the mixture centrifuged at 7500 × *g* (10 min, 4 °C). The aqueous phase was transferred to a fresh tube and the organic extraction was repeated twice. DNA was precipitated with 0.6 volume isopropyl alcohol (4 °C for 2 h). The DNA pellet was washed twice with 70% (v/v) ethanol and re-suspended in 3 mL TE buffer at 65 °C for 1 h, followed by addition of DNase-free Ribonuclease A (Sigma, St. Louis, MO, USA) to a final concentration of 20 μg ml^{-1} and incubated according to standard procedures. Following the RNase treatment, organic extraction was carried out twice on DNA as described above. The aqueous phase was transferred to a clean tube and DNA was precipitated by adding 0.1 volume of 3 M sodium acetate solution and 2 volumes of ethanol. The precipitated DNA was washed twice with 70% (v/v) ethanol, air dried for 20min, and finally resuspended in 500 μL TE.

DNA isolation from batch culture pellet containing plant debris and feed-particle associated bacteria (pellet A) was also performed using the CTAB extraction procedure described above. DNA extraction from fluid-associated bacteria (pellet B) was done using the Wizard® Genomic DNA Purification Kit (Promega Ltd, Madison, WI) and from supernatant B was performed using the QIAamp DNA Mini Kit (Qiagen Inc., Mississauga, ON), respectively.

2.2.6. DNA quantification and PCR

Extracted DNA was quantified spectrophotometrically and used for PCR. PCR amplification of a 540 bp fragment of *Brassica napus* ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (Rbc), a low copy nuclear-encoded gene, was used as control to detect endogenous canola DNA (GenBank Accession No. X75334). It was detected using forward primer (Rbc F) 5'-GCG TGA CGT CGT CAC GTA G- 3' and reverse primer (Rbc R) 5' -CGT TGC CTG CCA CAG GAT TAA GG- 3'. PCR conditions used were: 95 °C for 5min,

30 cycles of 94°C for 20s, 50°C for 30s, and 72°C for 3min, with a final extension of 72°C for 10min.

The *cp4 epsps* whole gene was detected using forward primer, EF1 (5' -TCA CGG TGC AAG CAG CCG TCC AGC- 3') and reverse primer, ER1 (5' -TCA AGC AGC CTT AGT GTC GGA GAG TTC G- 3') to amplify a 1363 bp region. PCR conditions used were: 94°C for 5min, 74°C for 5min, 35 cycles of 94°C for 1min, 74°C for 3min, and 72°C for 10 min.

Universal primers were used to detect bacterial DNA encoding 16S rDNA (Nadkarni et al. 2002) throughout the ruminal fermentation and from Pellet A. Forward primer, BF (5' -TCC TAC GGG AGG CAG CAG T- 3') and reverse primer, BR (5' -GGA CTA CCA GGG TAT CTA ATC CTG TT- 3') were used to amplify a 466 bp fragment as previously published (Nadkarni et al. 2002). PCR conditions were standardized with gradient PCR such that a 466 bp fragment of bacterial DNA was detected. The modified PCR conditions used were: 95°C for 10min, 22 cycles of 95°C for 15s, 68.5°C for 30s, and 72°C for 30s, followed by 72°C for 10min.

All PCR reactions (50 μL) contained the following (final concentrations): 1× PCR Buffer, 0.2 mM dNTP mix, 0.5 μM each of forward and reverse primer, 1.5 mM $MgCl_2$, and 2.5 U *Taq* Polymerase (Invitrogen, Burlington, Canada). 100 ng DNA template was used for PCR. A negative control without template DNA, as well as appropriate controls using non-transgenic leaf (PL) and/or transgenic leaf (RL) was included in each run. PCR was performed on a PTC 100 thermocycler (M.J. Research Inc.,Watertown, MA).

2.2.7. Gel electrophoresis

Five μL of genomic DNA isolated from canola substrates was applied to a 0.7% (w/v) agarose gel (Sambrook et al. 1989). For all PCR reactions, 20 μL of PCR products were analyzed on 1.5% (w/v) agarose gels.

2.2.8. Statistical anlaysis

VFA production data for each substrate was analyzed by ANOVA (SAS Institute 1999). The treatment effects were compared against each other using LS MEANS with PDIFF procedure.

2.3. RESULTS AND DISCUSSION

2.3.1. DNA isolation and PCR

DNA from canola leaf tissues was used as controls for all PCR analyses. DNA extraction from the seeds, meals, and diets was found to be of the best quality when the described CTAB extraction method was used and therefore this procedure was used to isolate DNA for batch culture samples. Initial attempts at PCR amplifications of DNA from the batch culture showed that 100 ng template DNA produced good amplification and was therefore used for PCR set-up.

2.3.2. Canola processing

Genomic DNA isolated from seeds (PWS and RWS) contained high molecular weight (approximately 23 kb) (Figure 2.1) and that from the canola meals (PCM and RCM) was highly fragmented but still had significant amounts of 23-kb DNA, while those from diets (PD and RD) contained comparatively lower amount of high molecular weight DNA. As expected, these results confirm that feed processing degrades plant DNA, most likely due to high temperatures during the processing. It has been reported previously that the treatment of oilseed rape meal (canola) resulted in complete degradation of DNA and that heating maize grains to 95°C for 5 min resulted in an inability to amplify a 577-bp gene sequence by PCR (Chiter et al. 2000). In the present study, during canola meal preparation, temperatures of 95°C were used and during diet processing temperatures of 100°C were achieved. Despite this, we were able to detect high molecular weight DNA and could successfully amplify full length plant genes (*epsp* synthase) from mixed diets containing canola meal.

The 540-bp Rbc fragment (endogenous plant DNA), was detected in all substrates of P and R (Figure 2.2A), and the *cp4 epsps* gene (1363 bp) could be detected from Roundup® Ready leaf and derived substrates viz; RWS, RCM, RD (Figure 2.2B). Despite using the same amount of DNA (100 ng) for PCR reactions, the 1363-bp band from RCM was less intense than that from RRC derived leaf and seed DNA, and amplification from RD DNA was even lower. These results are expected and suggest that fragmentation of DNA (transgene or otherwise) occurs during feed processing. In this case processing decreases the amount of 1363 bp DNA fragment in meals and diets. The diets contained 6.5% (w/w; as fed) of the respective canola meals and thus the transgenic DNA to total canola DNA ratio in RD would be expected to be lower, explaining the faint *cp4 epsps* band observed in diet RD (Figure 2.2B, Lane 9) as compared to meal RCM (Figure 2.2B, Lane 8). This reduction could have also arisen as a result of fragmentation of *cp4 epsps* during diet preparation. Presence of the *cp4 epsps* fragment in the diets was tested at least

three times from three different samples. The results indicate, that endogenous (Rbc) and recombinant plant genes (cp4 *epsp* synthase) are present and can be detected from processed canola diets.

2.3.3. Batch culture incubations

Total VFA concentrations were found to increase throughout fermentation for all the substrates tested, indicating continuous digestion of plant material (Figure 2.3). Within each treatment of GM or parental canola (whole seeds, cracked seeds, meals and diets), VFA concentrations did not differ significantly ($P > 0.05$). Acceptance of GM crops is dependent on substantial equivalence between the parental and transgenic lines (Martens 2000). Bioavailability of nutrients is a key component when comparing the equivalence of two feeds. We found that, *in vitro* digestion of parental and R substrates appeared equivalent, in terms of digestive end-products, which account for a major proportion of absorbable energy for ruminants.

Mean values of whole seed VFA were significantly lower than cracked seeds ($P<0.05$). This is accounted by the fact that the seed coat acts as a barrier to digestion of plant material which explains the observation that cracking the seeds resulted in a greater accumulation of fermentation end-products (Wang et al. 1997). VFAs from both whole and cracked seeds were significantly lower ($P<0.05$) than meals and diets, while differences between meal and diet values were not significant ($P>0.05$). Thus processing of meals and diets may have improved digestibility by increasing the plant material susceptible to microbial degradation.

Neither the endogenous 540 bp Rbc nor the recombinant 1363 bp *cp4 epsps* fragments were detected in supernatants (supernatant B) of batch culture incubations. However, bacterial DNA, was present in these samples (data not shown). Because nuclease activity is present in rumen fluid (Flint and Thomson 1990; Ruiz et al. 2000), we initially speculated that free plant DNA in the supernatant may have been degraded between the time taken for sampling and isolation of DNA. Thus batch culture incubations were repeated and DNA from the various fractions was isolated immediately (without a time lag). Plant DNA, however could still not be detected suggesting that plant DNA does not accumulate in the fluid fraction of rumen contents. The extremely high concentration of bacterial cells in rumen fluid compared to plant cells is perhaps the reason for detection of bacterial DNA in supernatant samples. Despite degradation of DNA by nucleases, the starting amount of bacterial DNA upon cell lysis must be high enough such that it can be amplified. On the other hand, plant DNA released into the aqueous environment from rumen fluid seems to be a small proportion of total DNA present. These results

are supported by the observation from another study which showed that the 1914 bp cry1A(b) maize gene persisted for less than one minute in rumen fluid and a 350 bp *bla* fragment survived for only 1 min (Duggan et al. 2000).

Persistence of endogenous plant DNA (Rbc) isolated from pellets containing plant debris (pellet A) was consistently the same within each treatment group (Figure 2.4), as was substrate digestion, indicated by VFA concentrations (discussed earlier). The 540-bp sequence was amplifiable for all time points from PW, RW, PC, RC, for up to 8 h in PCM and RCM, and for up to 4 h in PD and RD (Figure 2.4, A to H). These results indicate that plant DNA persistence is inversely related to plant cell digestion. Plant DNA from whole and cracked seeds could be detected for the longest incubation time, while total VFA production was expectedly the lowest for these substrates, as expected. Although mean VFA values did not significantly differ between meals and diets, the *Rbc* gene was detected for a longer incubation time in the meals (8 h) than in diets (4 h for PD and RD), which is most likely due to lower concentration of canola meal as well as degradation of canola meal in the diets. Similar trends were seen for the 1363-bp *cp4 epsps* whole gene in R substrates where the complete gene was detected at all time points in the whole and cracked seed samples, for up to 8h in the RCM, and for up to 4 h in the RD (Figure 2.5, A to D). Both *Rbc* and *cp4 epsps* are low copy genes and their equal lengths of detection in RCM and RD indicates similar rates of degradation.

The 466-bp bacterial-specific sequence could be amplified from fluid-associated bacteria (pellet B) throughout the 48-h incubation for all substrates whereas Rbc was not detected. The 466-bp fragment was also detected at all time points in the DNA isolated from pellet A, which contained plant material and particle-associated bacteria (data shown for PCM, PD in Figure 2.6A; and RCM, RD in Figure 2.6B). The detection of bacterial DNA in the 48-h incubation in meals and diets suggests that DNA isolated throughout the incubation was of PCR-quality and corroborates the fact that plant DNA was not present in detectable amounts past 8 h in meal and 4 h in diet preparations. These results also indicate that transformation of bacteria by the fragments of endogenous (*Rbc*) and recombinant (*cp4 epsps*) plant DNA studied here did not occur, despite transformation by fragments of DNA of this length being plausible (Dubnau 1999).

Transformation by DNA fragments can take place only if certain barriers are overcome as discussed previously (Ochman et al. 2000). First, DNA must be accessible to competent microorganisms. This would seem to be an initial limitation in the ruminant system since we found upon release, DNA is rapidly degraded. A second barrier is successful incorporation of foreign DNA into the competent recipient cells. It is well documented that recombination events are highly dependent on sequence similarity (Majewski 2001) and the newly acquired DNA must

be of use to the recipient cells. Stable transformation of ruminant microbes by recombinant DNA found in R canola was not detected in the present investigations and seems unlikely at least up to 48 h. However, transfer of genes across kingdoms within the rumen ecosystem has previously been demonstrated (Garcia-Vallve et al. 2000). What appears to be the determining factor then, is the balance between the high number of microbes in rumen fluid, to the number constantly in direct contact with plant material. This critical balance could reduce the effect of rapid DNA degradation acting as a barrier against transformation.

Despite this study being an analysis of plant DNA throughout in vitro incubation, the use of intact plant substrates instead of naked DNA provides important information on the fate of endogenous and recombinant canola DNA in a ruminal environment. Detection of plant DNA in the pellets containing plant debris, but not in the supernatant fluids, suggests that presence of plant DNA in ruminant systems is directly related to intact plant cells. Essentially, disappearance of plant DNA fragments is analogous to the digestion of plant cells in the rumen. This seemingly is the limiting step for transformation of rumen bacteria because once DNA is released into the aqueous ruminal environment, it is degraded almost immediately. Plant DNA fragments reaching the intestinal tract of ruminants and being available for absorption there (Einspanier et al. 2001) are most likely the result of undigested plant material passing through the rumen to the intestines, where plant cells can then be lysed, releasing DNA. The presence of intact plant genes in diets fed to ruminants warrants studies of DNA fragments in vivo and we are currently addressing this issue in trials with sheep fitted with ruminal and duodenal cannulae.

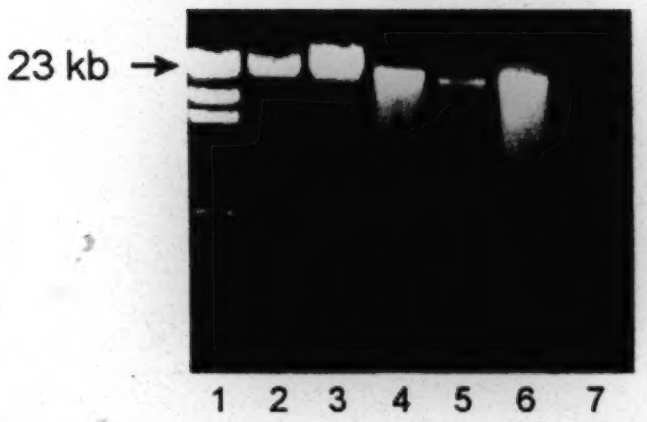

Figure 2.1 Gel electrophoresis (0.7% w/v agarose gel; 5 μL DNA per lane) of genomic DNA isolated by modified CTAB extraction from parental (P) and glyphosate-tolerant (Roundup Ready®, R) canola whole seeds, meals and diets derived from the meals. Lane 1: lambda DNA/*Hind*III molecular mass marker; Lane 2: Parental canola whole seed (PW); Lane 3: R canola whole seed (RW); Lane 4: Parental canola meal (PCM); Lane 5: Parental canola diet (PD); Lane 6: R canola meal (RCM); Lane 7: R canola diet (RD).

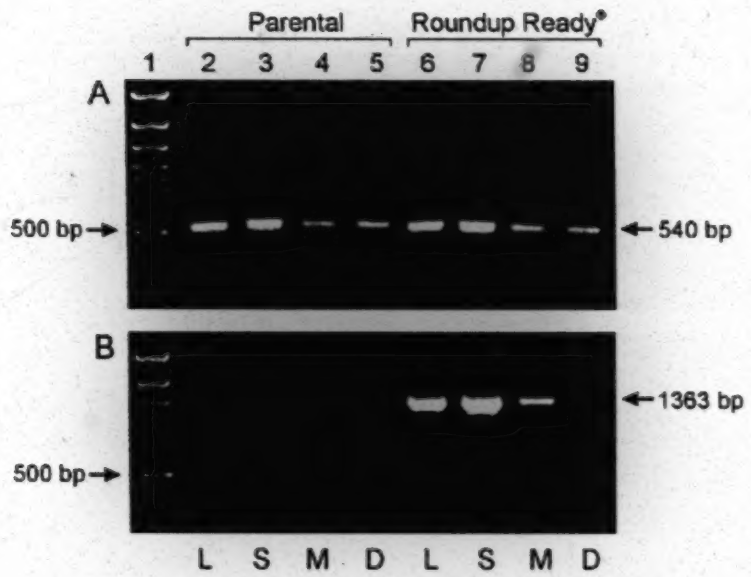

Figure 2.2 PCR analysis of DNA fragments from parental (P) and glyphosate-tolerant (Roundup Ready®, R) canola leaf (L), seeds (S), meals (M) and diets (D) derived from the meals. A. Presence of 540 bp canola-specific Ribulose-1, 5-bisphosphate carboxylase oxygenase (Rbc) fragment. Lane 1: 100 bp DNA ladder Plus; Lane 2: P leaf; Lane 3: P seed; Lane 4: P meal; Lane 5: P diet; Lane 6: R leaf; Lane 7: R seed; Lane 8: R meal; Lane 9: R diet. B. Analysis of 1,363 bp *cp4 epsps* transgene. Lane 1: 100 bp DNA ladder; Lane 2: P leaf; Lane 3: P seed; Lane 4: P meal; Lane 5: P diet; Lane 6: R leaf, Lane 7: R seed; Lane 8: R meal, Lane 9: R diet.

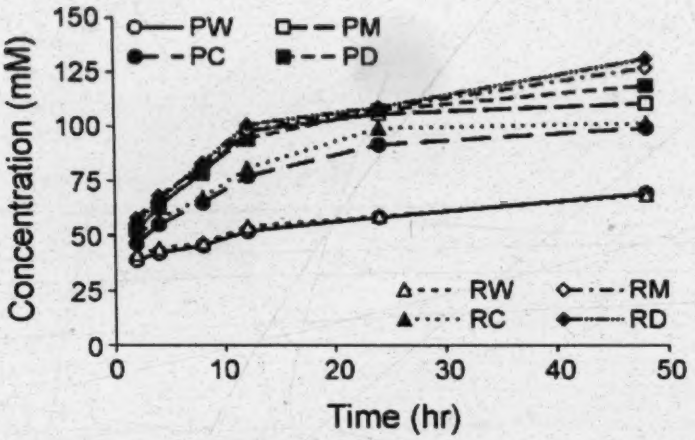

Figure 2.3 Total volatile fatty acid production during ruminal batch culture incubations of parental (P) and Roundup Ready® (R) canola whole seeds (W), cracked seed (C), meal (M), and diet (D). Each point represents an average of the concentrations from triplicate vials.

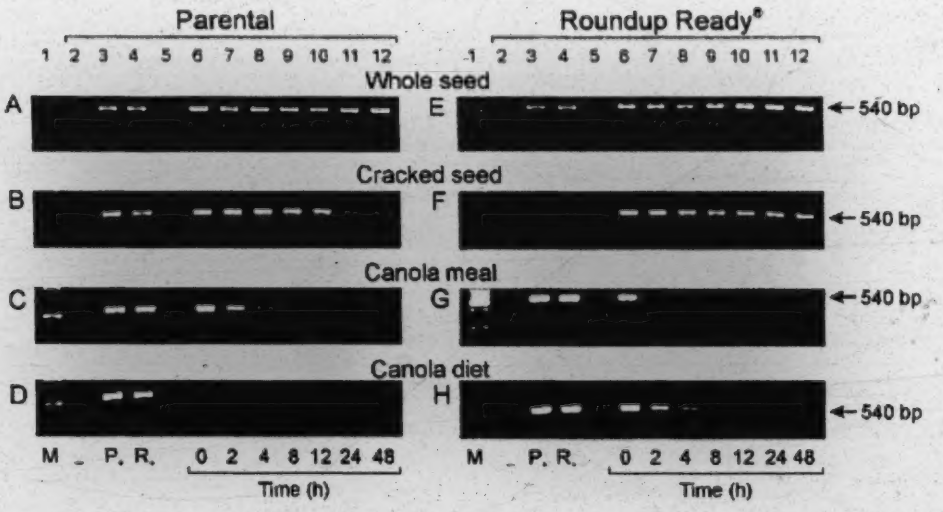

Figure 2.4 PCR analysis for persistence of 540-bp canola-specific Ribulose-1,5-bisphosphate carboxylase/oxygenase (*Rbc)* fragment throughout a ruminal batch culture incubation with parental (P) and Roundup Ready® (R) canola whole seed (A and E), cracked seed (B and F), meal (C and G) and diet (D and H). Lane 1:100-bp DNA ladder Plus (M); Lane 2: negative control contained all components of the PCR reaction except DNA template (-); Lane 3: P leaf positive control (P_+); Lane 4: R leaf positive control (R_+); Lane 6-12: represents time points 0, 2, 4, 8, 12, 24, and 48 h.

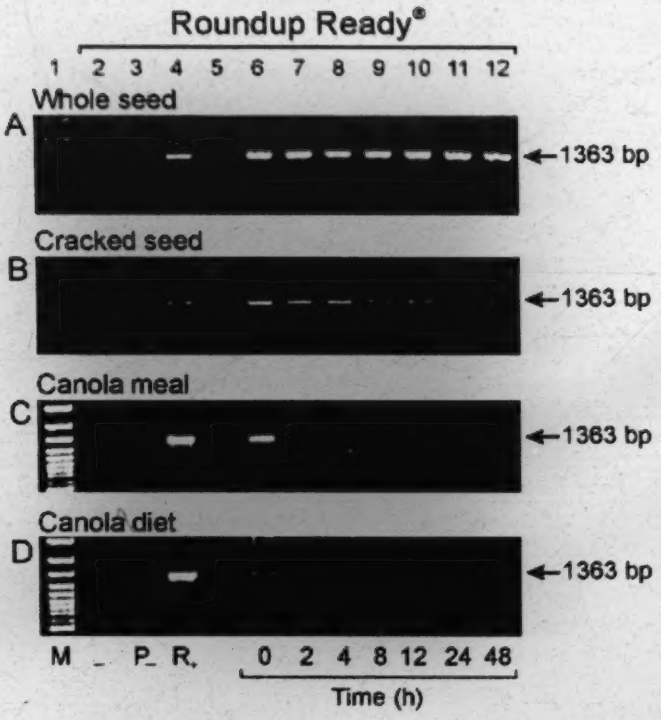

Figure 2.5 Detection of 1363-bp *cp4 epsps* transgene from ruminal batch culture incubation with Roundup Ready® (R) canola whole seed, cracked seed, meal and diet. Lane 1: 1-kb DNA ladder (M); Lane 2: negative control contained all components of the PCR reaction except DNA template (-); Lane 3: Parental leaf negative control (P_+) ; Lane 4: R leaf positive control (R_+); Lane 6-12: represent time points 0, 2, 4, 8, 12, 24, and 48 h.

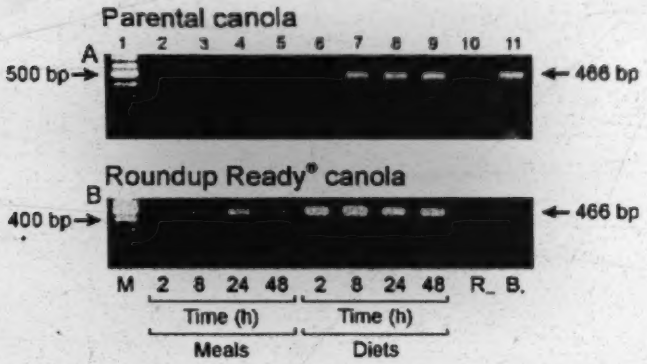

Figure 2.6 PCR amplification of a 466-bp bacterial-specific fragment from feed particle-associated bacteria obtained from ruminal batch culture incubations with: A. Parental (P) and B. Roundup Ready® (R) canola meals and diets. Lane 1: 100-bp DNA ladder Plus; Lanes 2-5: P or R meals at 2, 8, 24, and 48 h time points; Lanes 6-9: P or R diets at 2, 8, 24, and 48 h time points; Lane 10: R leaf negative control (R-); Lane 11: *E. coli* 466-bp fragment positive control (B_+).

2.4. REFERENCES

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Chapter 3 – Relative Stability and Fate of Transgenic DNA Fragments in Mixed Ruminal Cultures

3.1. INTRODUCTION

The agronomic improvements afforded by genetic modification of crops have resulted in a dramatic increase in their use in Canada, United States and Argentina (Kleter and Kuiper, 2002). Approximately 55% of the 12 million canola acres in Canada were seeded to genetically-modified (GM) varieties in 2000 (Canola Council of Canada 2001). In 2001, 256,000 tonnes of canola meal were used in ruminant production in Canada (Statistics Canada 2003). Thus, as adoption of GM crops increases, so too will their consumption by food animals. However, despite rigorous approval standards for transgenic plants, little is known about the fate of recombinant DNA following ingestion of GM crops by animals and exposure of the plant DNA to microorganisms within the digestive tract.

Ruminants harbour numerous bacteria, fungi and protozoa in their rumen and lower digestive tract, and even though these animals consume large amounts of plant material each day, most of the intact DNA present in their digesta is of microbial origin (Smith & McAllan, 1970). It has been shown that naked DNA is rapidly degraded in ruminal fluid (Duggan et al. 2000; Ruiz et al. 2000). From a study in which glyphosate-tolerant (Roundup Ready®) canola seeds and meal were incubated in bovine ruminal fluid, Alexander et al. (2002) concluded that any intact plant DNA found in the digesta was likely contained within intact plant cells, as plant DNA (endogenous or recombinant) was detected only in the feed particle fraction of the incubation medium. Similarly, in detecting Rubisco fragments in the spleen and liver of the soybean-fed mice, Hohlweg & Doerfler (2001) proposed that plant-associated DNA is more stable in the intestinal tract of mice than is naked DNA.

Rapid ruminal degradation of naked DNA would presumably reduce the potential for absorbance of genetic material across the intestinal wall. In the few studies with ruminants fed GM plant material, no full-length genes or large fragments of recombinant plant DNA were detected in animal tissues or products (Einspanier et al. 2001; Phipps et al. 2002). Einspanier et al. (2001) did, however, detect short fragments of endogenous plant DNA in the lymphocytes of cows fed GM maize. As well, Duggan et al. (2003) recently reported detection of a 211-bp amplicon from the *cryIA(b)* gene in ruminal fluid 24 h after feeding GM maize grain to sheep.

Transformation of gut microorganisms with recombinant DNA, or its absorption by

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animals consuming GM feeds may be size- or sequence-dependent. Given the abundance of nuclease activity in the rumen, it is likely that plant DNA released into that environment will be digested into a multitude of fragments of varying sizes, which may exhibit differential stability in the digesta. Presumably, fragments with greatest stability would more likely be taken up by bacteria or pass through the rumen to the lower digestive tract. The present study was conducted to investigate the persistence and possible differential stability in ruminal fluid of fragments of the *cp4 epsps* transgene from Roundup Ready® canola as whole and cracked seeds, and in formulations typical for use as animal feed.

3.2. MATERIALS AND METHODS

3.2.1. Canola substrates

Roundup Ready® canola and the parental line from which it was derived (denoted R and P, respectively) were used in the in vitro incubation, each as whole seed (PWS, RWS), cracked seed (PCS, RCS), canola meal (PCM, RCM) and a pelleted diet containing canola meal (PD, RD). Canola seed was obtained from Monsanto Company, St. Louis, MO, and canola meals were prepared at Texas Engineering Experiment Station, Texas A&M University, College Station, TX as described by Alexander et al. (2002). Canola plants were grown in a phytotron facility at the Lethbridge Research Centre to provide leaf tissue for DNA controls. Also at Lethbridge, barley grain-based pelleted diets for lambs were prepared that contained 65 kg of canola meal (PCM or RCM) per tonne. A rolling pin was used to crack whole seeds to produce PCS and RCS. Canola meals and diets were ground to pass through a 1-mm screen prior to use as substrates.

3.2.2. In vitro incubation

Inoculum for the in vitro incubation was prepared using ruminal content from a cannulated Jersey steer maintained on fresh alfalfa forage. At the barn, ruminal content (approximately 2 l) was strained through four layers of cheesecloth into a pre-warmed flask and the resulting solids were sealed into a plastic bag, and both were transported immediately to the laboratory. Inoculum was prepared by processing 1.5 L of ruminal filtrate and 375 g of solids in a blender (three 45-s pulses), straining the homogenate through four layers of cheesecloth, and combining the filtrate with two volumes of pre-warmed, pre-gassed buffer (Menke et al. 1979).

The incubation was conducted as described by Alexander et al. (2002). Briefly, 20 mL of

inoculum were added to 250 mg of canola substrate that had been pre-weighed into 35-mL serum vials. Triplicate vials of each substrate (PWS, RWS, PCS, RCS, PCM, RCM, PD, and RD) were prepared for each of seven sampling times (0, 2, 4, 8, 12, 24 and 48 h). Vials were flushed with CO_2 before and after addition of inoculum, then 18 vials of each substrate were sealed, affixed to a rotary shaker and placed in a 39°C incubator. The 0-h vials were processed immediately upon addition of inoculum, as described below.

3.2.3. Sample processing

Triplicate vials of each substrate were removed from the incubation after 0, 2, 4, 8, 12, 24, and 48 h. Progression of anaerobic fermentation in each vial was monitored by measuring gas production and accumulation of ammonia. Headspace gas was measured by water displacement (Fedorak and Hrudey 1983) prior to opening the vials, then the contents were transferred to 50-mL centrifuge tubes. Plant debris and particle-associated bacteria were sedimented by low-speed centrifugation (500 × *g*; 10 min; 4°C), following which the supernatant was decanted and the pellet (Pellet A) was frozen immediately in liquid N_2 for DNA isolation (below). A 2-mL aliquot of supernatant (Supernatant A) was set aside for determination of ammonia (Broderick and Kang 1980) and the remainder was immediately re-centrifuged (10,000 × *g*; 10 min; room temperature). The pellet (Pellet B), which comprised primarily fluid-associated bacteria, was flash frozen in liquid N_2 and the supernatant (Supernatant B) was processed immediately for isolation of any free DNA as described below.

3.2.4. Extraction of DNA

Isolation of free DNA from Supernatant B was conducted using a QIAmp DNA Minikit (Qiagen Inc., Mississauga, ON) and the manufacturer's protocol for body fluid samples. A modified CTAB extraction procedure (Alexander et al. 2002) was used for DNA extractions from Pellet A (i.e., plant debris; particle-associated bacteria), as well as from non-incubated whole and cracked canola seeds, canola meals, and diets. For isolation of DNA from Pellet B (fluid-associated bacteria), the Wizard® genomic DNA purification kit (Promega Ltd., Madison, WI) was used.

Canola plants (parental and Roundup Ready®) were grown in the phytotron facility at the Lethbridge Research Centre from seed from the same lot used for the in vitro incubation. Leaves from these plants were flash frozen in liquid N_2 immediately upon harvest, and the DNeasy Plant

Mini Kit (Qiagen Inc.) was used to extract DNA for inclusion as positive controls in PCR.

3.2.5. PCR analyses

Primer sets were designed to amplify seven different regions spanning the recombinant construct encoding *cp4 epsps* (Figure 3.1). Forward primer PF2 (5'- AAG GCA TTC ATT CCC ATT TG-3') and reverse primer ER3 (5'- ATT GCA GAT TCT GCT AAC TTG-3') amplified a 179-bp fragment (F_1) located in the promoter /CTP region of the construct; forward primer PF2 and reverse primer ER1 (5'-TAA CAT CTT CAC CTT CCA AAA G-3') amplified a 527-bp fragment (F_2) in the promoter/*epsps* region; forward primer EF6 (5'-GAC GCA GCA GCA TCC AC-3') and reverse primer ER1 amplified a 300-bp fragment (F_3) in the CTP/*epsps* region; forward primer EF4 (5'-CAA CAC TGG TAA GGC TAT GC-3') and reverse primer ER4 (5'-GGT AAC TGG AAG ACG ATC AC-3') amplified a 300-bp fragment (F_4) from the *epsps* region; forward primer EF5 (5'-CGT GGC TGA CTT GCG TG-3') and reverse primer ER5 (5'-CGT TAC CGA GAC CCT TAC C-3') amplified a 278-bp fragment (F_5) from *epsps*; forward primer EF2 (5'-TTG ATT GCG ATG AAG GTG AG-3') and reverse primer TR (5'-ACA AAT GGT ACA AGA AAA ACA G-3') amplified a 420-bp fragment (F_6) in the *epsps*/terminator region; forward primer EF2 and reverse primer ER2 (5'-TCA AGC AGC CTT AGT GTC G-3') amplified a 270-bp fragment (F_7) in the 3' *epsps* region. Conditions of PCR used for detection of the transgene fragments were the same for each of the primer pairs: 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 58°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min.

A 180-bp fragment of the gene encoding *Brassica napus* Rubisco small subunit, a low copy nuclear-encoded gene, was used as a control for detecting endogenous canola DNA (GenBank Accession No. X75334). It was detected using the forward primer *Rbc* F1 (5'-CAC ATA TCC ATG CGA TGC G-3') and reverse primer *Rbc* R1 (5'-ACC CAA AGA TAA AGG TAG CC-3'). Conditions of PCR for amplification of the *Rubisco* fragment were: 95°C for 5 min, 30 cycles of 94°C for 1 min, 62°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. Universal primers were used to detect bacterial DNA encoding 16S rDNA (Nadkarni et al. 2002) at each sampling time. Forward primer, BF (5'- TCC TAC GGG AGG CAG CAG T-3') and reverse primer, BR (5'-GGA CTA CCA GGG TAT CTA ATC CTG TT- 3') were used to amplify 466-bp fragment with thermocycling conditions of 95°C for 10 min, 22 cycles of 95°C for 15 s, 68.5°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min (Alexander et al. 2002).

All PCR mixtures (50 μL) contained the following (final concentrations): 1× PCR buffer, 0.2 mM dNTP mix, 0.5 μM each of forward and reverse primer, 1.5 mM $MgCl_2$, and 2.5 U *Taq* Polymerase (Invitrogen, Burlington, ON). For DNA isolated from plant (seeds, meals, diets, pellet from slow-speed centrifugation) or bacterial (*Escherichia coli* control, pellet from high-speed centrifugation) materials, 100 ng of DNA template was used in the PCR. However, the concentration of DNA isolated from the supernatant was well below 100 ng/μL, therefore 5 μL of DNA solution were used as template for PCR amplification. All PCR were performed on a PTC 100 thermocycler (M.J. Research Inc., Watertown, MA). Each PCR setup included a negative control (containing no template DNA), as well as appropriate positive controls (parental and/or transgenic canola leaf DNA as plant controls, and/or *E. coli* DNA as a bacterial control) as indicated. In all cases, 20 μL of PCR product were resolved on 1.5% (w/v) agarose gels containing ethidium bromide, using standard procedures (Sambrook et al. 1989).

3.2.6. Confirmation of limits of detection

Limit of detection assays were conducted to determine the sensitivity of the PCR for each of the primer sets designed to amplify the *cp4 epsps* transgene fragments. Purified Roundup Ready® canola meal (RCM) DNA was included in the 50-μL PCR mixtures in quantities of 1000, 500, 200, 100, 50, 25, 12.5, 6.25 and 0 pg. Matrix DNA isolated from the plant debris (Pellet A) from the 8-h parental diet (PD) incubation was included in each PCR (100 ng) as a control for template DNA. Other PCR mixture components, thermocycling conditions, and resolution of PCR products were identical to those described above.

3.2.7. Statistical analyses

Ammonia and gas production data were analyzed by ANOVA (SAS Institute, 1999). Treatment effects were compared using LS MEANS with PDIFF.

3.3. RESULTS AND DISCUSSION

3.3.1. Substrate digestion

Accumulations of gas and ammonia measured in the incubation vials over the 48-h incubation period (Figure 3.2) were indicative of active ruminal fermentation and consistent with

other reported findings for similar incubations (Wang et al. 1997). As expected, these parameters differed among substrate types (whole seed, cracked seed, meals and diets). Gas production at 48 h was strongly linked to the degree to which the substrates had been physically processed (D > M > CS > WS; $P < 0.01$), which reflects enhanced availability of digestible internal components of the canola seed (and barley grain, in the case of PD and RD) to microbial colonization and degradation (Wang et al. 1997). Ammonia concentrations at 48 h were also related to the degree of processing of the canola, but the effect was less pronounced (M > CS > WS and D; $P < 0.10$), because they arise as the balance of microbial amino acid deamination and microbial ammonia uptake (Wallace et al. 1997). The relatively low ammonia concentrations measured during incubation of diets (similar to WS; $P > 0.10$) is likely due to the lower crude protein content in the diets as compared with canola seed and meal.

Gas production and ammonia accumulation differed predictably among substrate types, but they were similar ($P > 0.05$) between parental and Roundup Ready® canola. This suggests that the presence of the *cp4 epsps* transgene did not affect ruminal degradation of the canola, i.e., that P and R were responding similarly to microbial digestive attack. The concept of substantial equivalence between a parental line of feed and its recombinant counterpart is a key feature in the acceptance of genetically-modified feeds (OECD 1993; Martens 2000).

3.3.2. Detection of endogenous and recombinant canola DNA

Endogenous canola DNA (represented by the 180-bp Rubisco fragment) was readily detectable in the pelleted plant debris (Pellet A). The duration of its persistence was related to the physical condition of the substrates, but no differences were evident between parental and Roundup Ready® canola (Figure 3.3). The Rubisco fragment was detectable at all time points in incubations of PWS, RWS, PCS and RCS (24 and 48 h not shown), but only at 0, 2, 4, and 8 h from meals (PCM and RCM) and not beyond 4 h in diets (PD and RD).

Persistence of *cp4 epsps* fragments during the in vitro incubation mirrored that of the endogenous 180-bp *Rubisco* fragment. Each of the seven fragments in DNA isolated from whole or cracked seeds was detectable at 48 h (Figure 3.4), whereas from meal and diets, excepting F_2 detectable in meal at 12 h, the fragments were not observed beyond 8 and 4 h, respectively (Figure 3.5). Limits of detection of the PCR assay was established at 12.5 pg (representative data presented in Figure 3.6). As with endogenous canola DNA and the complete 1363-bp transgene (Alexander et al. 2002), decline of *cp4 epsps* fragment concentrations below detectable limits by 8 h in incubations of meal-containing diet (RD), compared with 12 h in incubations of meal

(RCM), is attributed to lower initial concentration of canola DNA in the diet (6.5% meal; w/w) than in the meal itself. Observation of a faint F_2 band at 12 h in RCM was surprising, given that neither F_1 nor F_3, which both fall within F_2, was detected beyond 8 h. It may be that F_2 was present at a concentration near the limit of detection, and detected by chance alone at 12 h.

Patterns of persistence of endogenous (*Rubisco*) and transgene (*cp4 epsps*) DNA among whole, cracked, and meal-processed canola seeds were consistent with the determinations of in vitro digestibility (measured as accumulation of gas and ammonia; Figure 3.2). As digestibility of the canola preparation increased, the duration of persistence of DNA in the plant debris pellet decreased. No differences were observed between endogenous parental and Roundup Ready® canola DNA (*Rubisco* fragment), nor among the seven *cp4 epsps* fragments in the recombinant canola. In addition, relative persistence of the *cp4 epsps* fragments in whole, cracked and ground canola seed was consistent with observations made on the complete *cp4 epsps* transgene (Alexander et al. 2002). These observations suggest that endogenous and recombinant Roundup Ready® canola seed are substantially equivalent in terms of degradation of DNA during ruminal incubation, and that susceptibility of the transgene to degradation is essentially uniform along its length. Einspanier et al. (2001) attributed detection of a 199-bp large subunit *Rubisco* fragment in tissues of forage-fed cattle to high copy number of the endogenous DNA (as compared with a recombinant gene, which was not detected). The present findings support that conclusion, rather than a differential persistence of the 199-bp *Rubisco* fragment in the digesta.

Plant debris was evident (as Pellet A) throughout the 48-h incubation of meals and diets, yet the recombinant gene fragments were not detectable beyond 8 or 4 h, which is consistent with our earlier conclusion that DNA in breached plant cells is rapidly degraded by nucleases present in ruminal fluid (Alexander et al. 2002). McAllan and Smith (1973) reported similar degradation of plant cellular structures during in vitro ruminal incubation, evidenced as increasing concentrations of mono- and oligonucleotides in cell-free extracts from a 6-h incubation of alfalfa hay in ruminal fluid. That study did not distinguish between plant and bacterial DNA, thus it is possible that the increase in DNA may have been due to bacterial proliferation and lysis.

As expected, bacterial DNA (as the 466-bp 16S rDNA fragment) was detectable over the entire 48-h incubation in Pellet B (sedimented fluid-associated bacteria) from all eight substrates, whereas no canola DNA was found (data not shown). Bacterial DNA was also detected in all preparations of Pellet A (which included feed particle-associated bacteria), though as discussed above, canola DNA was only amplifiable for up to 8 h. In Supernatant B, bacterial DNA was detected consistently (Figure 3.7A), whereas endogenous (*Rubisco*) and recombinant (*cp4 epsps*) canola DNA were not (Figure 3.7, B to F).

To determine whether or not PCR inhibitors may have been present in the supernatant and limiting transgene detection, the PCR was repeated with a subset of mixtures randomly spiked with 50 ng of DNA isolated from Roundup Ready® canola leaf. In all cases, *Rubisco* and *cp4 epsps* sequences were readily amplified, confirming their true absence in supernatant.

The genes studied here are low or single copy, with *Rubisco* being nuclearly encoded, and the *cp4 epsps* construct being present at one copy per cell (Health Canada, 1999), which may explain why no plant DNA was detected in the supernatant after incubation. Another possibility is that upon lysis of plant cells in the ruminal environment, the DNA released is almost immediately degraded to sizes smaller than investigated here (less than 179 bp). Duggan et al. (2000) reported that a 350-bp recombinant gene sequence from maize chromosomal DNA could not be amplified beyond the first minute of incubation in ovine ruminal fluid. Detection of bacterial DNA in Supernatant B in the present study may be due to the high initial concentration of bacteria, such that even at an equally rapid rate of DNA degradation, the abundant template enabled its detection.

Knowing whether or not ruminal bacteria are capable of natural transformation and integration of recombinant plant DNA within the time frame (4 to 8 h) during which they could be exposed to the recombinant canola DNA is of interest, given that bacterial DNA was detected consistently in all fractions of ruminal fluid. Transfer of DNA to ruminal bacteria by transformation, conjugation, and transduction under laboratory conditions has been documented (Morrison 1996). In addition, although information on DNA exchange within the in vivo ruminal environment is lacking, there is some evidence that such processes have occurred across species (Nikolich et al. 1994; Garcia-Vallve et al. 2000).

The loss of biological activity of plasmid DNA within one minute of incubation in ruminal fluid (Duggan et al. 2000) suggests that duration of exposure of the DNA is likely a major constraint to such a transformation event occurring in rumen bacteria. Transformation of fluid-associated bacteria is unlikely in light of the short half-life of free DNA in ruminal fluid, but the close proximity to plant material of the feed particle-associated bacteria, which account for 70 to 80% of microbial matter in the rumen (McAllister et al. 1994), would increase the likelihood of their contacting plant DNA upon cellular degradation. A portion of these bacteria, formerly feed particle-associated, may be detected as fluid-associated following their release to the fluid milieu upon structural degradation of plant fragments (Cheng and McAllister, 1997). Had transformation by the recombinant DNA occurred, however, one or more of the *cp4 epsps* fragments ought to have been amplifiable either from Pellet A at time points beyond which *Rubisco* was no longer detectable, or from the bacterial preparations (Pellet B; Supernatant B).

Intact plant material rather than naked DNA was used in the in vitro incubations to mimic the animal feeding scenario, and has revealed uniformity of stability along the *cp4 epsps* transgene and no differences in ruminal stability between endogenous and recombinant plant DNA. This finding suggests that all of the primer sets examined in the present study should work equally well for detecting the presence of the *cp4 epsps* gene in a variety of matrices. On the basis of observations from the present study, future research will focus on culture and PCR analysis of feed particle-associated bacteria from early-stage in vivo incubations (i.e., within 4 h of feeding GM canola to livestock) – the conditions most favorable for a transformation event to occur.

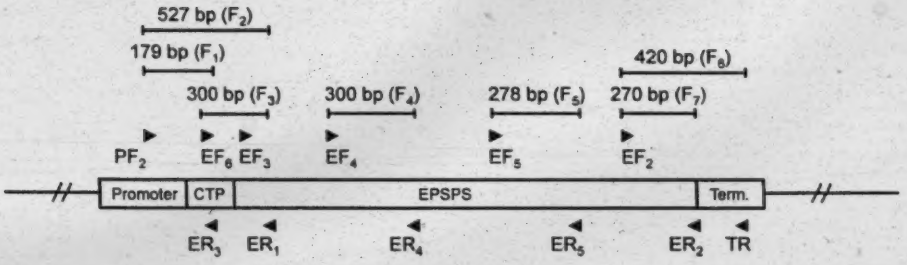

Figure 3.1 Schematic representation of the *cp4 epsps* construct and the position of the designed primers and the respective fragments amplified (F1 to F7). Drawing not to scale. F1 (179 bp) was amplified using primer set PF2/ER3, F2 (527 bp) with primer set PF2/ER1, F3 (300 bp) with primer set EF6/ER1, F4 (300 bp) with primer set EF4/ER4, F5 (278 bp) with primer set EF5/ER5, F6 (420 bp) with primer set EF2/TR, and F7 (270 bp) with primer set EF2/ER2.

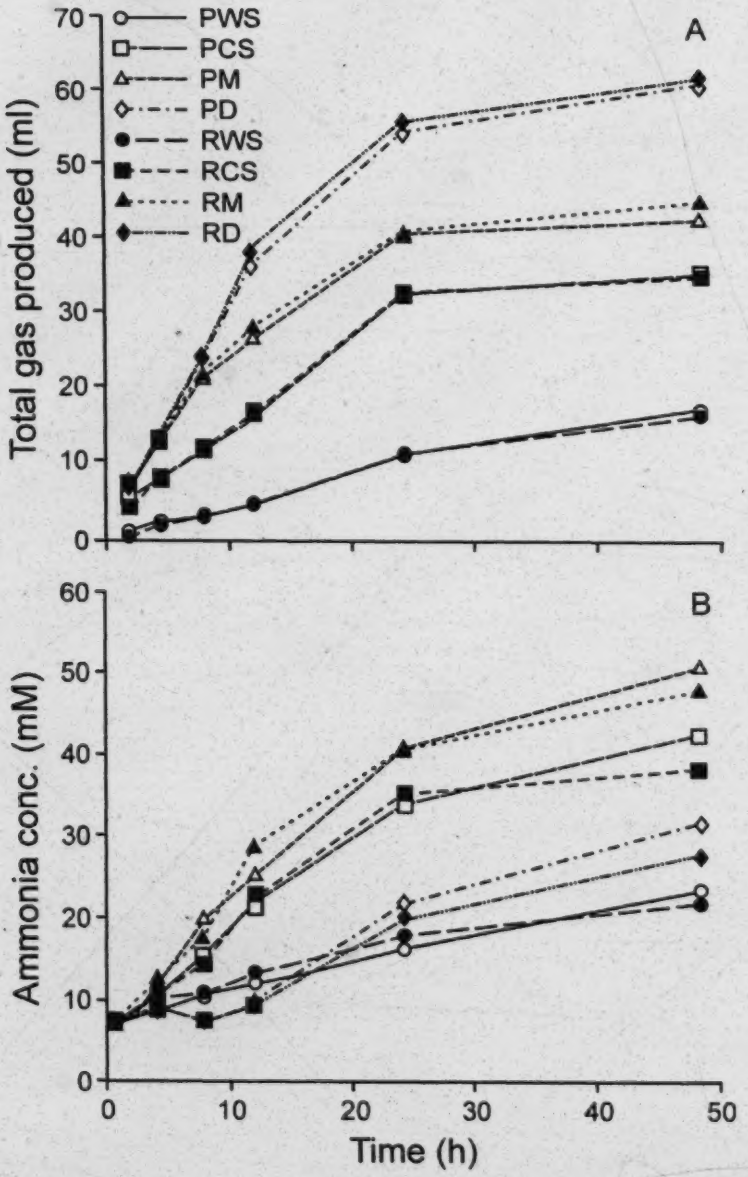

Figure 3.2 Total gas production (A) and ammonia accumulation (B) during ruminal batch culture incubations of parental (P) and Roundup Ready® (R) whole seeds (WS), cracked seed (CS), meal (M), and diet (D). Each point represents an average of the concentrations from triplicate vials.

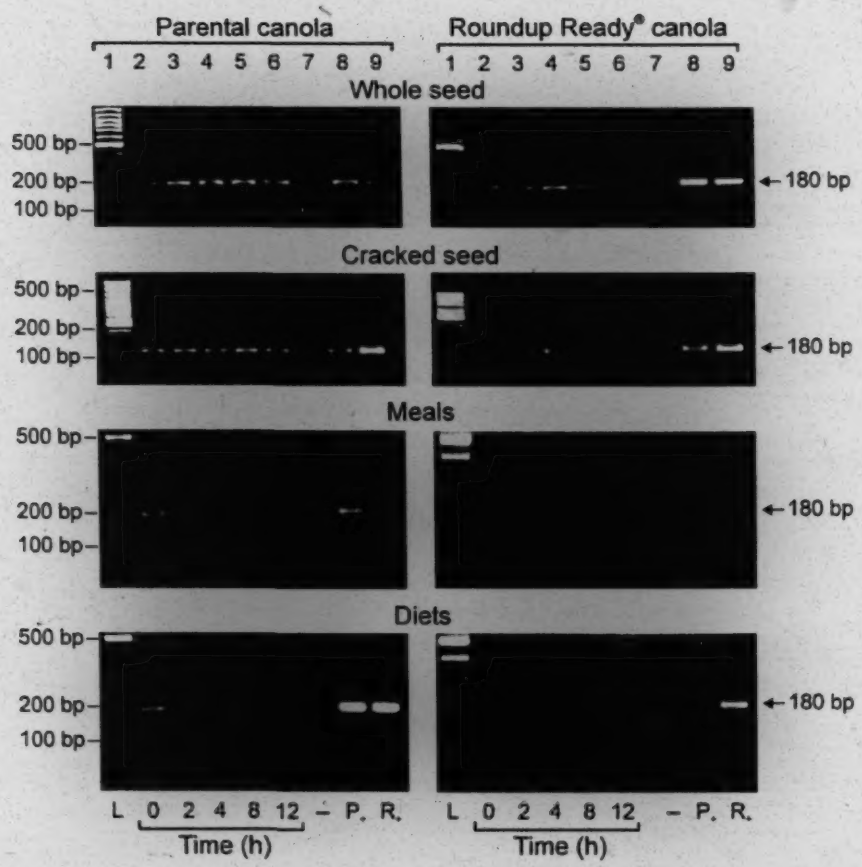

Figure 3.3 Detection of endogenous canola DNA (as a 180-bp canola-specific *Rubisco* fragment) in sedimented plant debris from the first 12 h of incubation of whole and processed parental and Roundup Ready® canola in buffered ruminal fluid. Lane 1: 100-bp DNA Ladder Plus (L); Lanes 2 to 6: DNA isolated from samples collected after 0, 2, 4, 8 and 12 h of incubation; Lane 7: negative control (no DNA template); Lanes 8 and 9: positive controls (DNA isolated from leaves of parental (P_+) and Roundup Ready® (R_+) canola grown from the whole seed).

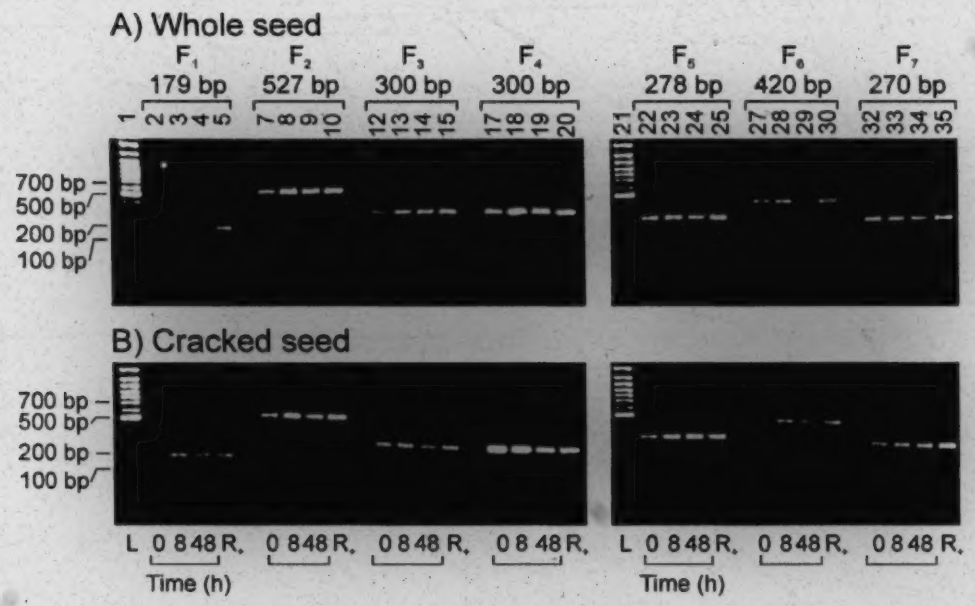

Figure 3.4 Detection of seven different *cp4 epsps* fragments spanning the transgene construct (see Fig. 3.1) in DNA isolated from sedimented plant debris from a 48-h incubation of (A) whole and (B) cracked Roundup Ready® canola seeds in buffered ruminal fluid. Lanes 1 and 21: 100-bp DNA Ladder Plus (L); Lanes 2 to 20 and 22 to 35: four-lane sets pertaining to each of the seven fragments (F_1 to F_7). They contain (left to right) DNA from samples collected after 0, 8 and 48 h of incubation, plus a positive control (R_+) which is DNA isolated from leaves of Roundup Ready® canola grown from the whole seed. Lanes 6, 11, 16, 26 and 31 are empty.

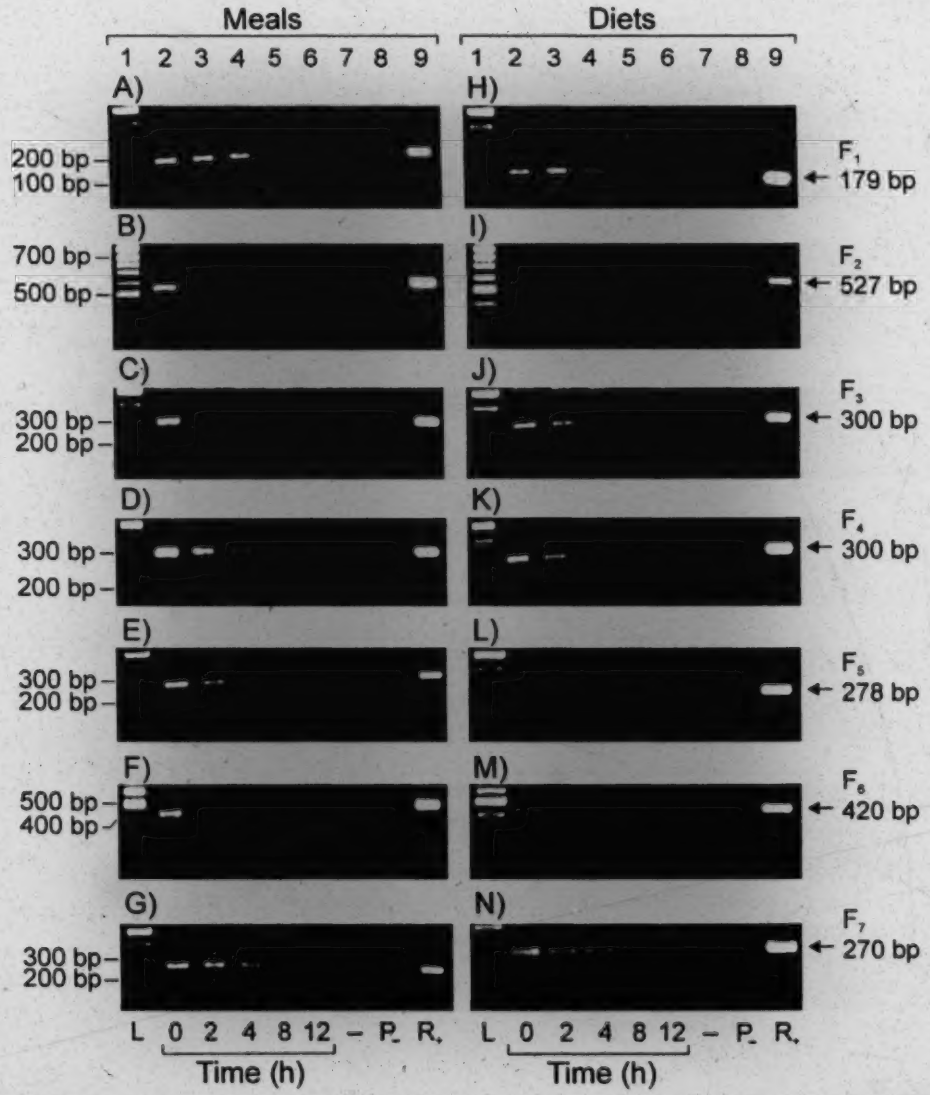

Figure 3.5 Detection of seven different *cp4 epsps* fragments (see Fig. 3.1) in sedimented plant debris from the first 12 h of incubation of meal (A-G) and pelleted diet (H-N) prepared from Roundup Ready® canola. Lane 1: 100-bp DNA Ladder Plus (L); Lanes 2 to 6: DNA from samples collected after 0, 2, 4, 8 and 12 of incubation; Lane 7: negative control (no DNA template); Lanes 8 and 9: positive controls (DNA isolated from leaves of parental (P_+) and Roundup Ready® (R_+) canola grown from seed.

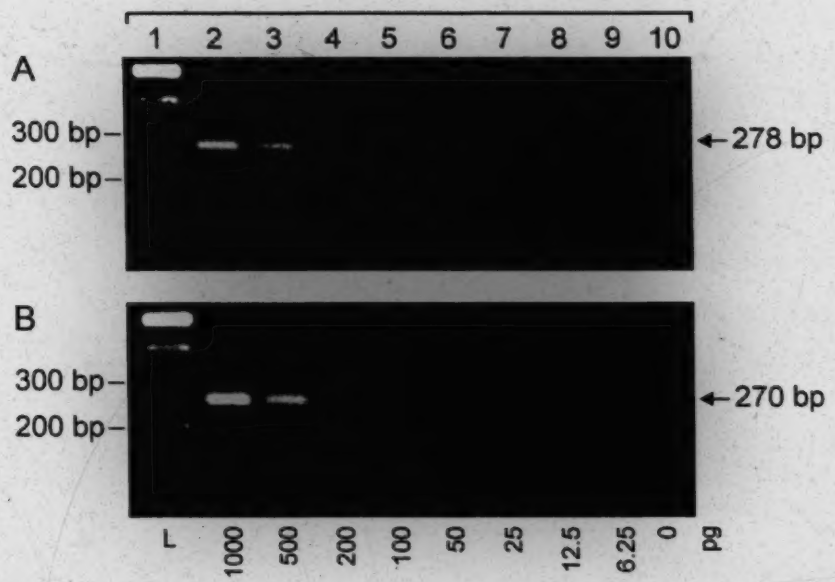

Figure 3.6 Demonstration of sensitivity of the PRC assay for detecting *cp4 epsps* fragments. PCR mixtures (containing 100 ng of DNA from 8-h parental meal Pellet A as a control for template DNA) were spiked with known quantities of DNA purified from Roundup Ready® canola meal. Assays were conducted with all seven primer sets. Representative data [F_5 in (A); F_7 in (B)] are shown. Lane 1: 100-bp DNA Ladder Plus (L); Lanes 2 to 10: PCR mixtures containing 1000, 500, 200, 100, 50, 25, 12.5, 6.25 or 0 pg of purified DNA from RCM.

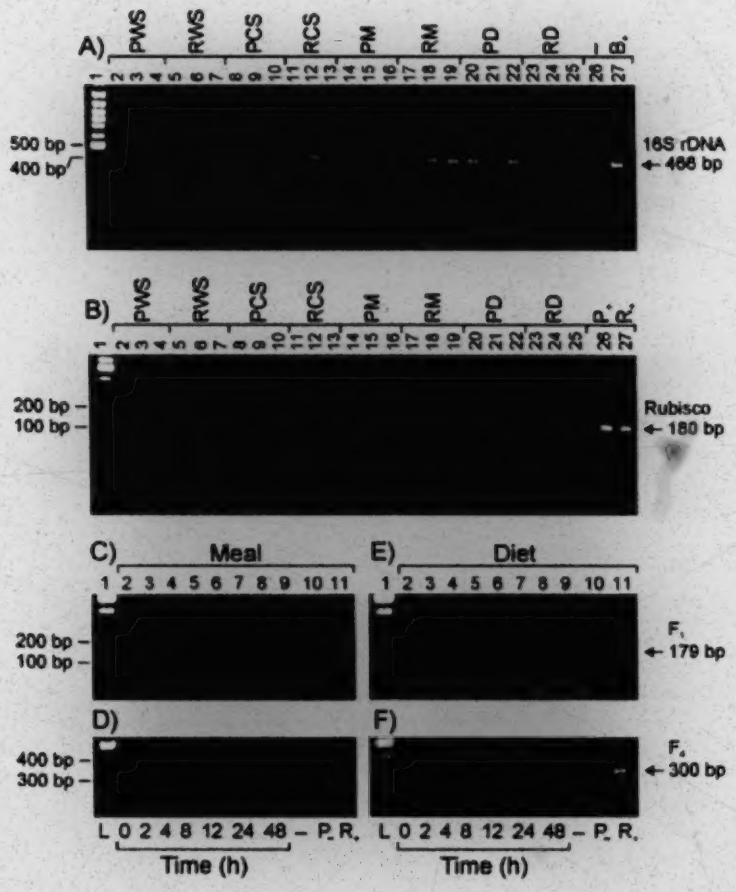

Figure 3.7 Detection of (A) bacterial DNA, as a 466-bp fragment of 16S rDNA, (B) endogenous canola DNA, as a 180-bp canola-specific *Rubisco* fragment, and (C to F) *cp4 epsps* DNA (F_1 and F_4 as representative data) in Supernatant B from 48-h in vitro incubations of parental (P) and Roundup Ready® (R) canola in buffered ruminal fluid. Each canola line (P and R) was incubated as whole seeds (PWS; RWS), cracked seeds (PCS; RCS), meal (PCM; RCM), or pelleted diet (PD; RD) made with canola meal. In (A) and (B), Lane 1: 100-bp DNA Ladder Plus (L); Lanes 2 to 25: three-lane sets pertaining to each of the eight substrates, containing (left to right) DNA from samples collected after 0, 8 and 48 h of incubation; Lane 26: negative control (no DNA template); Lane 27: positive control (466-bp fragment amplified from *E. coli*). In (C) to (F), Lane 1: 100-bp DNA Ladder Plus (L); Lanes 2 to 8: DNA from samples collected after 0, 2, 4, 8, 12, 24, and 48 h of incubation; Lane 9: negative control (no DNA template); Lanes 10 and 11: DNA from leaves of parental and Roundup Ready® canola, serving as negative (P_-) and positive (R_+) controls for *cp4 epsps*.

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Chapter 4 – Use of Quantitative Real-time PCR to Assess the Stability of the *cp4 epsps* Transgene from Roundup Ready® Canola in the Intestinal, Ruminal and Fecal Contents of Sheep

4.1. INTRODUCTION

The area of arable land dedicated to genetically-modified (GM) plants has steadily increased over the last seven years, reaching a total area of 58.7 million hectares worldwide in 2002 (James 2002). The expanding use of GM plants as food sources for livestock and humans has increased the scrutiny as to whether there are environmental or adventitious presence issues associated with GM crops. To a large part, regulations by most countries ensure that GM plants entering the food chain do not have any negative health effects. A series of nutritional studies have reported no adverse health or production effects in animals consuming GM feeds (Hammond et al. 1996; Sidhu et al. 2000; Barriere et al. 2001; Aumaitre et al. 2002). Regardless of these findings, there still is merit in determining the fate of recombinant plant DNA and expressed proteins within agricultural environments. In fact, attention given to GM plants over the last few years has led to some fundamental questions regarding the fate of plant DNA within and between kingdoms (de Vries et al. 2001; Einspanier et al. 2001; Thomson 2001). The stability of transgenic plant DNA throughout animal digestive tracts is one such area of interest.

Canola meal, produced after oil extraction, is an important protein supplement for livestock animals. Our lab has previously characterized the effect of feed processing on the stability of transgenic and endogenous canola plant DNA within the rumen (Alexander et al. 2002). It seems that the majority of plant DNA in the rumen is housed within intact plant cells, as it is rapidly degraded upon exposure to ruminal nucleases as a result of plant cell lysis. Others have reported that naked plant and animal DNA are rapidly degraded in ruminal fluid, and plasmid DNA loses the capacity to transform bacteria within one minute of incubation in ruminal fluid (Duggan et al. 2000; Ruiz et al. 2000). Given that the transit time of the fluid fraction of ruminal contents to reach the small intestine is greater than one hour, it is likely that most high molecular weight plant DNA entering the small intestine of ruminants is in the form of undigested plant material. It does seem that there is potential for plant DNA to be absorbed along the digestive tract of ruminants, as fragments of high-copy endogenous plant DNA have been detected in blood lymphocytes of cattle (Einspanier et al. 2001). Transgenic DNA could not be detected along with the endogenous sequences, suggesting that the low copy number of transgenic inserts limits their chance for absorption or detection. In mice, absorption of DNA

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fragments has been localized to the intestinal wall and Peyer's Patches (Schubbert et al. 1997). If this is where absorption of DNA fragments is to take place in ruminants, then lysis of plant cells would have to occur somewhere within the lumen of the small intestine in order for DNA to be available for absorption.

Roundup Ready® (R) canola is tolerant to the glyphosate family of herbicides by expressing transgenic DNA from the CP4 strain of *Agrobacterium tumefaciens*, encoding 5-enolpyruvylshikimate-3-phosphate synthase protein (*cp4 epsps*). The purpose of this study was two-fold: first, to examine the stability of free plant DNA in ruminal, duodenal, and fecal fluids isolated from sheep; and secondly, to use quantitative real-time PCR to assess the stability of the *cp4 epsps* transgene in R canola meal during incubation with digesta from the small intestine of sheep. Our lab has published data (Alexander et al. 2002; Sharma et al. 2004) showing that Roundup Ready® canola (event GT73) and the parental line it is derived from, are digested similarly in sheep digesta contents and that endogenous DNA from both lines behave similarly. Therefore, the focus of these experiments was to study the stability of transgenic DNA only.

4.2. MATERIALS AND METHODS

4.2.1. DNA, diets, and animals

Roundup Ready® canola seed (event GT73) and canola meal were supplied by Monsanto Company, St. Louis, MO, USA. The canola meal was prepared at Texas Engineering Experiment Station, Texas A&M University (TX, USA). The diet used in this study contained 15% (w/w; as fed) canola meal on an as-fed basis and was prepared at the Lethbridge Research Centre, Lethbridge, AB, Canada. Both the meal and diet have previously been shown to contain fragments of DNA approximately 23-Kbp in size after processing and the entire 1363-bp *cp4 epsps* transgene and construct regions flanking it are amplifiable by PCR (Sharma et al. 2004; Alexander et al. 2002).

For the free DNA incubation experiment, DNA was extracted from R canola seed using a CTAB method described earlier (Alexander et al. 2002). For the batch culture incubation experiment, the diet was first ground to pass through a 1-mm screen. To prevent possible contamination of digesta by R canola or soybean-containing diets, the five ruminally and duodenally fistulated Canadian Arcott wethers used in each of the experiments described in this study were fed alfalfa hay only (ad libitum intake). Animals used in this study were cared for according to the guidelines set by the Canadian Council of Animal Care (CCAC 1993).

4.2.2. Stability of free DNA in digesta fluids

Ruminal fluid, duodenal fluid, and feces were sampled from five sheep 4 to 5 h after feeding. An equal quantity of each digestive sample was collected from all of the animals and pooled together. For ruminal and duodenal contents, the samples were processed under anaerobic conditions. Approximately 100 mL of fluid from the proximal duodenum was collected from each animal by fastening a 100-mL whirl-pack bag (NASCO, Modesto, CA, USA) onto a simple T-type cannula. Ruminal contents were obtained through use of a syringe fitted with a hose 2-cm in diameter. Fecal matter was collected by digital palpation directly from the rectum of sheep.

Ruminal and duodenal fluids were passed through four layers of cheesecloth. The filtrate from duodenal fluid was separated into three equal volumes: the pH of two of the aliquots was adjusted to 5 and 7 by addition of sodium hydroxide (6 M) while the third was left at the original pH (3.2). Fecal matter was diluted in a 1:9 ratio (w/v) with 1× phosphate-buffered saline (Sambrook et al. 1989) and vortexed. This mixture and the ruminal and duodenal filtrates were the media used for incubations. Two micrograms of R canola DNA were added to 100 μL of each medium and incubated at 39°C for: 0, 0.5, 1, 2, 5, 10, 30, 60, 120, and 240 min. After each time point, 400 μL of Buffer AP1 from a Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON) was added to stop the reaction, and the mixture was processed according to the manufacturer's instructions. For time point 0 min, the buffer was first added to the medium, followed by the DNA.

4.2.3. Scanning electron microscopy

Whole duodenal and ruminal samples were collected from two sheep after the morning feeding as described above. The ruminal contents served as a positive control for feed-associated bacteria. Triplicate sub samples (2 mL) were prepared for scanning electron microscopy as described previously (McAllister et al. 1992), with the following modifications: three washes in sodium cacodylate buffer followed the prefixation. This was followed by a 2-h postfixation in 2% (w/v) osmium tetroxide in cacodylate buffer amended with 0.5% (w/v) ruthenium red. After postfixation, the samples were washed six times with water and then dehydrated with a graduated ethanol series to 100% (v/v) ethanol followed by critical point drying.

4.2.4. Stability of DNA during duodenal fluid batch cultures

Duodenal fluid was collected from five sheep as described above. The samples were pooled and blended for three sessions of 45 s under CO_2. This homogenate was filtered through four layers of cheesecloth and the strained fluid was added to three volumes of pre-warmed buffer (Menke et al. 1979). The buffer/duodenal fluid inoculum was divided into two equal volumes and adjusted to pH 5 and 7, respectively, using concentrated HCl. Each inoculant was dispensed (20 mL) into 35-mL serum vials containing 300 mg of the diet listed above. The vials were sealed and incubated at 39°C on a rotary shaker for 0, 1, 2, 4, 8, 12, and 16 h. At each time point, triplicate vials were withdrawn from the incubator, placed on ice, and then processed according to the procedure outlined by Alexander et al. (2002). Briefly, the complete contents of each vial were emptied into a 50-mL conical tube and centrifuged at 3200 × *g* for 10 min at 4°C. The supernatant was decanted and the remaining pellet (pellet A), containing plant debris and feed particle-associated bacteria, was frozen in liquid nitrogen for later DNA analysis. A 2-mL subsample of the supernatant (supernatant A) was pipetted into a 2-mL microcentrifuge tube and centrifuged at 13 000 × *g* for 4 min at room temperature. The resulting supernatant (plant cell-free, supernatant B) was transferred to another 2-mL tube and frozen in liquid nitrogen for DNA analysis. Total volatile fatty acid (VFA) concentrations from supernatant A were later determined by gas chromatography as described previously (McAllister et al. 1998).

Separate incubations were also conducted to determine the effect of pH on plant cell integrity and stability of the transgene encoding *cp4 epsps* protein. For this, two experiments were performed by incubating the diet in the previous buffer at pH 5 or 7 in the absence of duodenal fluid. To prevent bacterial growth, ampicillin was added to a final concentration of 100 μg mL^{-1}. Microbial digestion was confirmed to be limited by the absence of gas production as measured by water displacement (Fedorak and Hrudey 1983). The first experiment entailed incubating triplicate samples of 300 mg of diet in buffer at each pH for 0, 4, 8, and 12 h. The samples were centrifuged at 3200 × *g* for 10 min at 4°C, and the resulting pellet was frozen in liquid nitrogen and stored for DNA extraction at a later time. The second experiment determined dry matter (DM) disappearance of quadruplicate 500-mg samples of the diet after 0, 4, 8, and 12 h of incubation. At each time point, the plant material in the vials was collected on a pre-weighed 0.45-μm filter and then dried at 110°C for 24 h to estimate DM disappearance.

4.2.5. DNA isolation

The DNA incubated in digesta fluids as described in section 4.2.2. was extracted by use

of a Qiagen DNeasy Tissue Kit. Isolation of DNA from pellet A (section 4.2.4.) was performed as described by Alexander et al. (2002) with the following modifications: the pellets were first lyophilized and ground in dry ice with a mortar and pestle. The ground material was then incubated in 6 mL of CTAB lysis buffer, followed by chloroform/isoamyl alcohol (24:1) extraction. After the last step, 200 μL of DNA in 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) was mixed with 350 μL of Buffer AP3/E and processed from step 7 onwards according to the Qiagen DNeasy Plant Mini Kit instructions. This entailed filtering the DNA/Buffer mixture through a DNeasy mini spin column, washing the column with Buffer AW, and elution of DNA from the column with preheated (65°C) 1× TE. Isolation of DNA from supernatant B was performed as follows: 1 mL of the supernatant was extracted three times with an equal volume of chloroform/isoamyl alcohol, followed by precipitation in 0.8 volume of isopropyl alcohol, washing with 70% (v/v) ethanol, and resuspension in 50 μL of TE buffer. Extracted DNA was quantified spectrophotometrically.

4.2.6. PCR analyses

Primer sets used for PCR and the regions of DNA they amplified are described in Table 4.1. Five primer pairs (F1, R1 – F5, R5) designed to amplify DNA sequences (SF1, SF2, SF3, SF4, and LF) spanning the transgenic construct were used to detect R canola throughout naked DNA incubations in digesta fluids. For fragments SF1, SF2, SF3, and SF4, PCR conditions were: 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s followed by annealing at 58°C for 30 s and extension at 72°C for 1 min; extension at 72°C for 10 min. Conditions for fragment LF were: 94°C for 5 min; 35 cycles of 94°C for 1 min and 74°C for 3 min; final extension at 72°C for 10 min.

Universal primers (F7, R7) were used to detect bacterial DNA (Fragment 16S, Table 4.1) encoding 16S rDNA (Nadkarni et al. 2002) from feed-associated bacteria in pellet A, described in section 4.2.4. Thermocycling conditions were: 94°C for 5 min; 30 cycles of 95°C for 15 s, 68.5°C for 30 s, and 72°C for 30 s; extension at 72°C for 10 min.

All conventional PCR reactions (50 μL) contained the following (final concentrations): 1× PCR buffer, 0.2 mM dNTP mix, 0.5 μM each forward and reverse primer, 1.5 mM $MgCl_2$, and 2.5 U *Taq* Polymerase (Invitrogen, Burlington, ON, Canada). One hundred nanograms of DNA template were used for PCR. A negative control without template DNA, as well as a positive control using DNA from R canola leaf tissue (plant analysis) or *Escherichia coli* (bacterial analysis), were included in each set of PCR analyses. PCR was performed with a DNA Engine

Dyad (M.J. Research Inc., Watertown, MA, USA). For each PCR, 20 μL of product was analyzed on a 1.5% (w/v) agarose gel. In order to assess the stability of different segments of transgenic DNA in digesta fluids, as well as different sizes of DNA, conventional PCR was used for analyses of DNA isolated as described above. Qualitative results were obtained. The time point immediately prior to loss of visual detection of a PCR product by agarose gel electrophoresis was considered the time to which that fragment persisted.

The primers (F6 and R6) and probe (Pr) used for quantitative real-time PCR were designed using Primer Express 2.0 Software (Applied Biosystems, Foster City, CA, USA), and amplified a 62-bp sequence (RT1, Table 4.1) spanning the chloroplast transit peptide/*cp4 epsps* coding region. The probe (Table 4.1) was labeled at the 5' end with the reporter dye 6-carboxyfluorescein (6-FAM) and at the 3' end with the quencher dye 6-carboxytetramethylrodamine (TAMRA). An ABI Prism 7700 Sequence Detection System (Applied Biosystems) was used for PCR. The conditions were 50°C for 2 min; 95°C for 10 min; and 45 cycles of 95°C for 15 s and 60°C for 1 min. All reactions contained (final concentrations): 1× Universal Master Mix (Applied Biosystems), 0.15 μM each primer, and 0.05 μM probe. The R canola event GT73 has one transgenic insert in its genome. Therefore, a quantitative standard curve using the ratio of one transgenic insert per 2C value of 2.45 pg was developed (Arumuganathan and Earle 1991). In each standard, a total of 245 ng of DNA from canola seed was present. The standards included 0, 2, 20, 200, 2.0×10^3, 2.0×10^4, 5.0×10^4, and 1.0×10^5 copies of transgenic insert per reaction. As transgenic canola was diluted, non-GM seed DNA (parental line to GT73) was substituted in its place so that the final concentration of canola DNA in each standard reaction did not change. For the unknown samples, 245 ng of DNA was also added to each reaction.

4.2.7. Statistical analyses

Dry weight, VFA, gas, and *cp4 epsps* copy number data were analyzed by the Proc Mixed procedure of SAS (SAS Institute 1999) using CS structure. Differences between means were considered significant if the *P*-values were less than or equal to an alpha value of 0.05.

4.3. RESULTS AND DISCUSSION

4.3.1. Stability of free DNA in digesta fluids

The fragments of DNA studied ranged from 300 to 1363-bp in size and covered sequences within the *cp4 epsps* coding region as well as those spanning the junctions of *cp4 epsps* with other elements of the insert. There were clear differences in the stability of transgenic canola DNA depending on the digestive site, and in the case of the small intestine, the pH of the fluid. Persistence of DNA was longest in duodenal fluid at pH 3.2 and in feces, with detection of the largest fragment, LF, occurring even at 240 and 120 min, respectively, whereas the smaller fragments, SF1-SF4, were detectable for 120 and 240 min (Figure 4.1, Table 4.2). All fragments of DNA persisted for 10 min in duodenal fluid at pH 5 and in ruminal fluid. In duodenal fluid at pH 7, DNA was the least stable, with fragments SF1-SF4 not being amplified beyond 2 min and the 1363-bp *cp4 epsps* transgene not being detected after 0.5 min.

It has been reported that the rumen and large intestine account for 95% of the mean retention time of digesta in sheep and are therefore the major sites of feed digestion (de Vega et al. 1998). Stability of DNA in feces was greater than that in ruminal fluid, but to our knowledge, free transgenic plant DNA has not been detected in the feces of ruminants. Einspanier et al. (2001) detected no DNA encoding the *cry1Ab* transgene in the feces of cattle consuming Bt maize silage. However, transgenic DNA has been detected in the rectum of pigs up to 48 h after consuming Bt maize (Reuter and Aulrich 2003) and the Cry1Ab protein has been identified in the feces of calves consuming diets containing Bt maize kernels (Chowdhury et al. 2003). Furthermore, Netherwood et al. (2004) recently reported detection of the *cp4 epsps* transgene in digesta from the small bowel of ileostomized humans after consumption of a GM soya protein supplement. This shows that transgenes from feed and food are more likely to enter the lower digestive tract in the absence of foregut microbial fermentation. Factors such as the type of feed and particle size may also influence the quantity of transgene that reaches the lower tract. Nevertheless, the data presented here suggest that should plant DNA be liberated in the colon of sheep, it may be stable for up to 2 h.

In this study, none of the fragments of transgenic DNA was detected beyond 10 min of incubation in ruminal fluid. This is probably due to the high DNase activity present in ruminal fluid (Flint and Thomson 1990; Ruiz et al. 2000). The rapid degradation of DNA in the rumen, coupled with a lengthy turnover of the fluid fraction of ruminal digesta, suggests that it is unlikely that significant quantities of free transgenic plant DNA released in the rumen would reach the small intestine.

Characterizing the stability of transgenic DNA in duodenal fluid at three different pH values was designed to mimic conditions of exposure between the proximal and distal ends of the small intestine. Degradation was found to be greatest at pH 7, which coincides with the optimal

pH for activity of pancreatic nucleases and for favorable microbial growth. In its free form, the *cp4 epsps* transgene was considerably more stable in duodenal fluid at pH 3.2 as compared to pH 7, persisting for 240 and 0.5 min, respectively. In the small intestine, DNA is susceptible to endonucleases, which have an optimal pH in the range of 6.8 to 8.2 (Armstrong and Hutton 1974). Phosphodiesterase I and II, with optimal activities at pH 9.3 and 7, respectively, are also responsible for the degradation of nucleic acids in the small intestine.

The data presented here provide information on the factors affecting stability of free DNA in the small intestine. Persistence of naked DNA in the duodenum is dependent on flow rate as well as pH. In sheep, the pH of fluid in the proximal duodenum is typically between 2.6 and 3.0 (Merchen 1993). Nuclease activity appears to be minimal in this pH range as all fragments of the *cp4 epsps* transgene were detectable after incubation for 2 h (Figure 4.1, Table 4.2). However, the pH of intestinal fluid increases as it flows to the ileum and the present data suggest that this would be accompanied by an increase in the degradation of free transgenic DNA. Bicarbonate buffers from pancreatic secretions do not neutralize digesta until the mid-jejunum, 7 to 15 m distal to the pyloric sphincter (Ben-Ghedalia et al. 1974; Nicoletti et al. 1984). Neutralization likely requires between 25 min and 1 h, considering that the passage of digesta through the small intestine requires anywhere from 1.5 to 4.5 h (Coombe and Kay 1965; Grovum and Williams 1973). Consequently, although fragments of DNA may persist in the contents of the duodenum at low pH, they are unlikely to persist long enough to reach the most probable site of absorption at the Peyer's Patches in the ileum (Schubbert et al. 1997).

To date, studies analyzing the presence of transgenic plant DNA in animal tissues have either failed to detect fragments of target genes or have only been able to identify fragments of endogenous plant genes with a high copy number in the genome (Einspanier et al. 2001). Given that the GT73 canola event has a single insert of *cp4 epsps*, and that free DNA is rapidly degraded in intestinal fluid at pH 7, the likelihood of absorption of even small fragments of the *cp4 epsps* transgene seems remote.

4.3.2. Scanning electron microscopy

For all samples analyzed from the duodenum, bacterial colonization on the outer surfaces of feed particles was sparse (Figure 4.2A). In contrast, feed particles from the rumen were heavily colonized by bacteria (Figure 4.2B). It appears that the majority of surface-adherent biofilms are removed as a result of exposure to physical agitation within the acidic and proteolytic environment of the abomasum. However, microbial biofilms persisted within the crevices of feed

particles collected from the duodenum (Figure 4.2C) and were similar to those in the rumen (Figure 4.2D). The glycocalyx of the biofilms collected from feed particles in the duodenum were even more apparent than in feed particles collected from the rumen. The glycocalyx plays an integral role in bacterial attachment (Miron et al. 2001) and may offer some protection against environmental stressors (Dunne 2002). The increased production of glycocalyx may have been in response to stressors such as gastric juices and endogenous enzymes, and it may also help facilitate continued attachment of bacteria to feed particles as they move through the small intestine.

The limited stability of naked DNA added directly to ruminal fluid suggests that the appearance of large segments of free plant DNA in the small intestine would have to arise from lysis of intact plant cells at this point within the digestive tract. Feed particle-associated bacteria and fungi are capable of lysing plant cells, particularly when biofilms have become fully established (McAllister et al. 1994). The fact that intact digestive biofilms remain on feed particles in the small intestine raises the possibility that microorganisms may still continue to liberate plant DNA within the small intestine.

4.3.4. Stability of DNA from diets during batch cultures

Increased microbial growth in duodenal fluid at pH 7 as compared to pH 5 resulted in higher ($P < 0.01$) concentrations of VFA (Figure 4.3). In the rumen, microbial growth is impaired below pH 5.5 (Slyter et al. 1966), but some ruminal bacteria such as *Streptococcus bovis* (Russell et al. 1979) and *Lactobacillus* spp. tolerate acidic conditions and remain viable below pH 5 (Erfle et al. 1982). This study demonstrated that at least some of the microorganisms within the rumen are capable of withstanding passage through the abomasum and remain active within duodenal fluid.

Concentrations of VFA in the culture at pH 7 did not increase until after 4 h, indicating a significant lag time in microbial growth (Figure 4.3). In contrast, VFA production occurs rapidly in ruminal cultures (Alexander et al. 2002), which may reflect the higher bacterial numbers present in ruminal fluid as compared to duodenal contents (Nicoletti et al. 1984). It has been reported that bacterial numbers in intestinal digesta increase from the proximal to the distal small intestine (Harrison et al. 1971; Nicoletti et al. 1984). The delay in microbial growth observed in this study was likely exacerbated by the dilution of the intestinal fluid and, therefore, microbial activity was assessed over a 16-h period. Considering that retention of digesta in the small intestine of sheep is only 1.5 to 4.5 h, the contribution of intestinal bacteria to digestion is likely limited.

The profiles of individual VFA measured in this experiment differed from those seen in ruminal fluid (data not shown). Acetate and butyrate accounted for 97% of VFA produced and the acetate to propionate (A:P) ratio increased from 4:1 at 0 h to 28:1 at 16 h. In comparison, the A:P ratio in ruminal fluid is typically 3:1 (Merchen 1993). Others have reported a higher A:P ratio (37:1 to 107:1) in the ileum as compared to the rumen (Lewis and Dehority 1985). This suggests that flow of digesta from the rumen and the type of substrates available in the small intestine promotes the establishment of bacteria with a propensity to ferment carbohydrate to acetate. The fact that similar changes in the A:P ratio occurred in vitro as observed in vivo (Lewis and Dehority 1985) suggests that the lack of nutrient absorption in the in vitro model did not markedly alter the fermentative populations present.

The *cp4 epsps* copy number quantified from the solid plant material differed ($P < 0.05$) between pH 5 and 7 (Figure 4.4A). In aqueous duodenal fluid at pH 7, copy number of *cp4 epsps* reached a maximum of approximately 1600 at 8 h (Figure 4.4B), decreasing rapidly thereafter in a manner that coincides with heightened microbial activity as indicated by an increase in VFA concentration. No copies of the transgene were detected in fluid at pH 7 before 2 h or after 16 h of incubation. Similarly, at pH 5, the transgene was only detected between 2 and 12 h, but in this case the copy number never exceeded 20. The low copy number of *cp4 epsps* in the fluid fraction at pH 5 may be a reflection of the more gradual release of the transgene into the liquid phase over time. In contrast, the greater amounts of transgenic plant DNA detected at pH 7 were likely the result of the microbial lysis of a large number of plant cells and the simultaneous release of multiple copies of the transgene. Despite quantification of the 62-bp DNA sequence by real-time PCR, the 1363-bp *cp4 epsps* gene was never detected in the supernatant of either pH treatment (data not shown). This suggests that although release of plant DNA is possible within the small intestine of sheep, large sequences of DNA are highly unstable and are quickly degraded to smaller fragments. This relationship is supported by the in vitro incubations with naked DNA described in section 4.3.1.

In spite of limited microbial activity, *cp4 epsps* copy number in solid plant material declined more rapidly in duodenal fluid at pH 5 as compared to that at pH 7. At pH 7, the decline in *cp4 epsps* copy number was negatively correlated ($r = -0.98$) with VFA production, implicating the role of bacteria in degradation of the gene. The more rapid decline in *cp4 epsps* copy number at pH 5 is more difficult to explain but was repeatable in three separate experiments. Initially, we thought that the difference in pH may have affected the quantitative PCR procedure, but serial dilutions of DNA isolated from fluid at pH 5 and 7 resulted in a predictable decrease in the number of *cp4 epsps* copies detected (data not shown). Furthermore, PCR with a universal

probe for bacterial DNA showed that bacterial DNA increased in the pH 7 inoculant, but not in the pH 5 inoculant (data not shown). Therefore, the more rapid decline in the *cp4 epsps* copy number at pH 5 was not a result of bacterial growth, which was also reflected by minimal changes in VFA concentration.

There is evidence that a variety of cellulases and expansins exist in the cell wall and are capable of weakening it (Hager 2003). Many of these enzymes have optimal activity at pH 5 (Hayashi et al. 1984; Kotake et al. 2000; Tabuchi et al. 2001). Although possible, it is difficult to envision how these enzymes would have retained sufficient activity to cause cell lysis and expose the *cp4 epsps* transgene to degradation by nucleases. However, this hypothesis was supported by a decrease in DM and *cp4 epsps* transgene in buffer, which were higher at pH 5 than at pH 7 after 4 h (Figure 4.5, A and B). Dry matter loss and the decline in *cp4 epsps* copy number in buffer also correlated ($r = 0.91$ and $r = 0.99$, respectively) with the decline in copy number from solid digesta throughout incubation in duodenal fluid at pH 5. Gas production did not significantly increase throughout the incubations in buffer at pH 5 or pH 7, implying that bacterial digestion was minimal for both treatments (data not shown). Together, these data suggest that the main reason for a decrease in *cp4 epsps* copies throughout the duodenal cultures at pH 5 was probably due to the effect of acidity on plant cell stability. The pH of duodenal fluid was adjusted to 5 to approximate that of the mid-jejunum. Despite a large decline in transgenic DNA over a 16-h period (Figure 4.4), this study showed that most DNA within intact plant cells will be released into the ileum where the pH is more favorable for microbial growth.

In conclusion, the present study revealed that the stability of naked DNA varies throughout the ovine digestive tract. Our findings suggest that although degradation of plant material is possible in the small intestine, DNA fragments released from plant cells persist only for a short time, limiting the likelihood of their absorption. Using real-time PCR, digestion of the *cp4 epsps* transgene in canola was shown to be highly correlated with the digestive activity of intestinal microorganisms as indicated by increases in VFA concentration. This relationship may enable plant species-specific primers to be designed that would enable documentation of the differential digestion of plant cells within the digestive tract. The real-time PCR data also suggested that plant cell lysis and liberation of DNA might continue to occur even when the pH is not optimal for production or activity of fibrolytic enzymes by intestinal microorganisms.

Table 4.1 Description of primers and a probe used in the amplification of Roundup Ready®-specific plant DNA or universal bacterial DNA

Fragment name	Primer or probe sequence (5' to 3')	Target
Plant specific		
SF1[a] (527 bp)	(F1[b]) AAG GCA TTC ATT CCC ATT TG (R1[c]) TAA CAT CTT CAC CTT CCA AAA G	Promoter/*cp4 epsps*[d]
SF2[a] (300 bp)	(F2) GAC GCA GCA GCA TCC AC (R2) TAA CAT CTT CAC CTT CCA AAA G	Chloroplast transit peptide/*cp4 epsps*
SF3[a] (300bp)	(F3) CAA CAC TGG TAA GGC TAT GC (R3) GGT AAC TGG AAG ACG ATC AC	Within *cp4 epsps*
SF4[a] (420 bp)	(F4) TTG ATT GCG ATG AAG GTG AG (R4) ACA AAT GGT ACA AGA AAA ACA G	*cp4 epsps*/terminator
LF[e] (1363 bp)	(F5) TCA CGG TGC AAG CAG CCG TCC AGC (R5) TCA AGC AGC CTT AGT GTC GGA GAG TTC G	Entire *cp4 epsps* gene
RT1 (62 bp)	(F6) CCA CGG CGT GCA TGC (R6) ACC AGA GGA CTT ACG AGC AGT TG (Pr[f]) TCA CGG TGC AAG CAG CCG TCC	Chloroplast transit peptide/*cp4 epsps*
Bacteria specific		
16S[g] (466 bp)	(F7) TCC TAC GGG AGG CAG CAG T (R7) GGA CTA CCA GGG TAT CTA ATC CTG TT	16S rDNA

[a] From Sharma et al. (2004)

[b] Forward primer.

[c] Reverse primer.

[d] Transgene encoding the synthetic enzyme 5-enolpyruvylshikimate-3-phosphate synthase derived from *Agrobacterium* sp. strain CP4.

[e] From Alexander et al. (2002)

[f] Probe for real-time PCR.

[g] From Nadkarni et al. (2002)

Table 4.2 Persistence of recombinant DNA sequences spanning the synthetic *cp4 epsps* gene during incubation in digesta samples[a] from sheep

	Fragment[b]				
	SF1 527 bp	SF2 300 bp	SF3 300 bp	SF4 420 bp	LF 1363 bp
Ruminal fluid	10 min	10 min	10 min	10 min	10 min
Duodenal fluid (pH 3.2)	120 min	240 min	240 min	120 min	240 min
Duodenal fluid (pH 5)	10 min	10 min	10 min	10 min	10 min
Duodenal fluid (pH 7)	2 min	2 min	2 min	2 min	0.5 min
Feces	240 min	120 min	120 min	120 min	120 min

[a] Two micrograms of Roundup Ready® canola seed DNA were incubated in 100 µl of each sample. Ruminal and duodenal samples were undiluted filtrates from passage of fluids through four layers of cheesecloth. Fecal material was diluted 1:9 (w/v) with 1× phosphate-buffered saline. Duodenal fluid pH was adjusted to 5 or 7 by addition of sodium hydroxide.

[b] Fragments of DNA are described in Table 4.1.

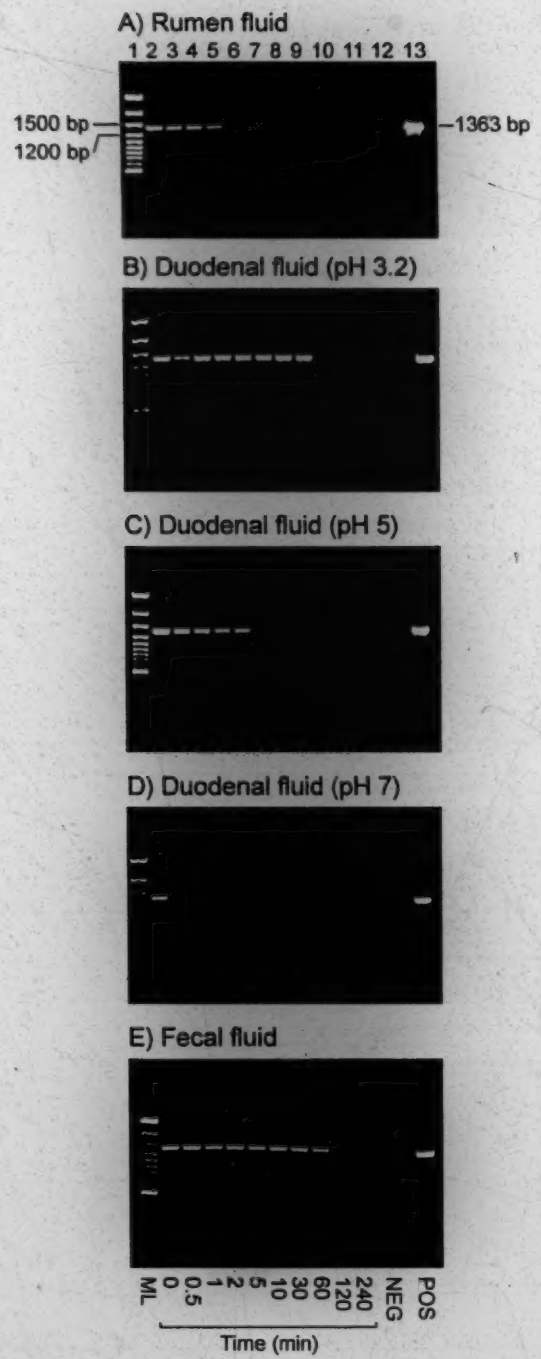

Figure 4.1 PCR analysis for bacterial DNA, endogenous canola DNA, and *cp4 epsps* recombinant DNA fragments in cell-free supernatant from 48-h in vitro incubations of parental and Roundup Ready® canola seeds, meals and diets in buffered ruminal fluid.

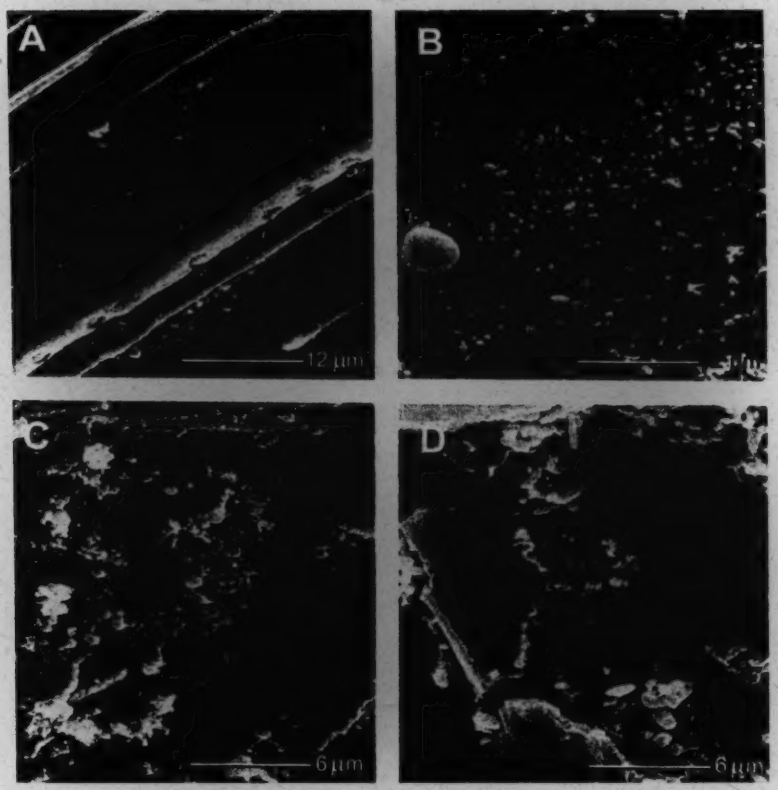

Figure 4.2 Scanning electron micrographs of feed particles isolated from the duodenum (A, C) and rumen (B, D) of sheep. Microbial colonization of surfaces (A, B) and perforations (C, D) of the particles are shown.

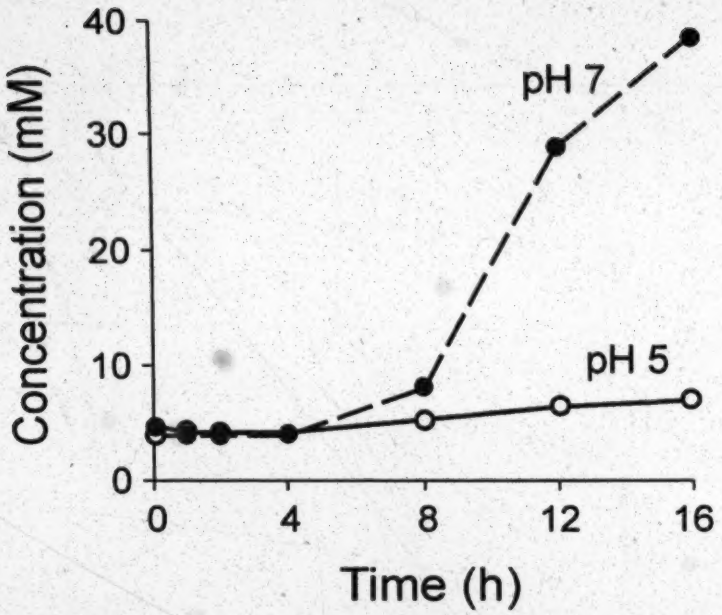

Figure 4.3 Total volatile fatty acid (VFA) measurements during batch culture incubations of a diet containing Roundup Ready® canola meal in whole duodenal fluid adjusted to either pH 5 or 7. Values shown are the averages plus standard error, n=3 per time point.

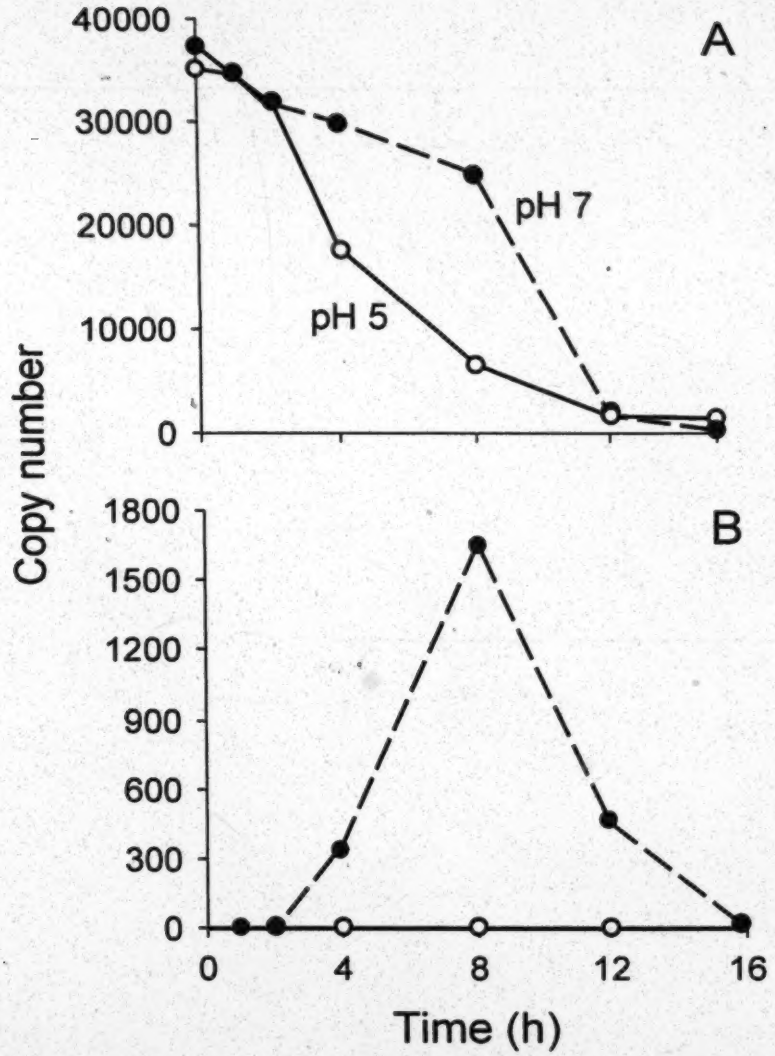

Figure 4.4 Real-time PCR analysis of the persistence of *cp4 epsps* throughout batch culture incubations of a diet containing Roundup Ready® canola meal in whole duodenal fluid adjusted to either pH 5 or 7. Values shown are the average copy number plus standard error, n = 3 per time point. A: Analysis of DNA isolated from the pellet of vial contents (3200 × *g*; 10 min). B: Analysis of DNA isolated from cell-free supernatant (13 000 × *g*; 4 min).

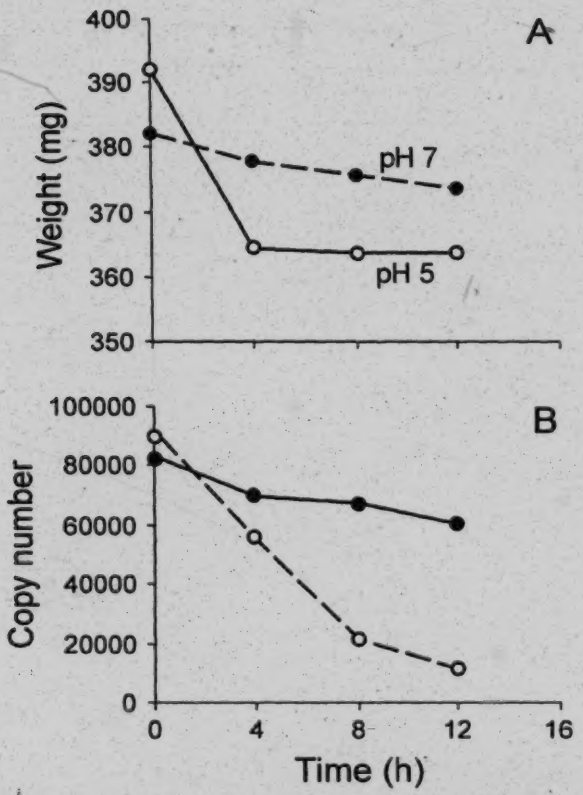

Figure 4.5 Recovery of dry matter (A) and persistence of *cp4 epsps* transgene (B) in diet containing meal from Roundup Ready® canola during 12 h of incubation in buffer at pH 5 (open symbols) or pH 7 (closed symbols). Ampicillin was included at 100 μg ml^{-1} to inhibit bacterial growth. Diet dry matter was collected on a 0.45-μm filter. Copy number was determined by real-time PCR on DNA isolated from pelleted material (3200 × g; 10 min). Average values from quadruplicate (A) or triplicate (B) determinations are shown. Bars indicate standard error.

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Chapter 5 – Conventional and Real-time PCR Assessment of the Fate of Transgenic DNA in Sheep Fed Roundup Ready® Canola Meal

5.1. INTRODUCTION

The attention given to studying genetically-modified (GM) plants has led to interesting findings pertaining to the detection, persistence, and survivability of specific plant genes and proteins in animal systems. Absorption of plant DNA across the intestinal barrier seems to be a natural event. Endogenous (native) plant genes have been detected in tissues and products from poultry (Tony et al., 2003; Klotz et al., 2003; Aeschbacher et al., 2005), swine (Reuter and Aulrich, 2003; Nemeth et al., 2004; Mazza et al., 2005), and cattle (Einsapnier et al., 2001; Nemeth et al., 2004), albeit to varying extent depending on gene copy number, DNA fragment size, and type of diet. Until recently however, recombinant plant DNA has not been found in animal tissues. Nemeth et al. (2004) detected a short fragment (123 bp) of transgenic DNA in swine tissue and our laboratory has detected transgenic DNA (278 bp) in the liver and kidney of swine (Sharma et al., 2006). In both studies, positive detection was a rare event. Recently, significantly larger fragments of endogenous (533 bp) and transgenic (519 bp) DNA have been reported with greater frequency in swine tissues (Mazza et al., 2005). In each of the studies reporting positive transgene detection, the appearance of transgenic DNA in animal tissues was not statistically more frequent than endogenous DNA.

The range of fragment sizes and discrepancies in occurrence of transgene detection in these studies warrant further investigation into the fate of transgenic DNA in animals. Because major interest lies in the detection of recombinant DNA in animal tissues and the transformation of bacteria by recombinant DNA, it is logical to investigate the amount of transgenic DNA available for each event. The likely place for absorption is the distal small intestine and proximal large intestine, specifically, via the Peyer's patches (Schubbert et al., 1997). A previous study has demonstrated that DNA liberated from plant cells is likely rapidly digested by endogenous and microbial nucleases within the small intestine of sheep (Alexander et al., 2004). Although detection of transgenic DNA has been reported throughout the digestive tract of cattle consuming GM feed, the amount of transgenic DNA reaching the small intestine of ruminants has not been reported. Einspanier et al. (2004) attempted to quantify the amount of transgene in digesta from cattle fed GM maize silage, however it was not possible due to the low levels of transgene after ensiling. Netherwood et al. (2004) were successful in quantifying the number of transgene copies

[a] This chapter has been submitted for publication. Br. J. Nutr. February, 2006.

passing to the small intestine of human ileostomists consuming GM soya, and found that up to 3.7% of transgene could survive passage.

Roundup Ready® (R) canola is rendered tolerant to the glyphosate family of herbicides through expression of transgenic DNA from the CP4 strain of *Agrobacterium tumefaciens* that encodes 5-enolpyruvylshikimate-3-phosphate synthase (*cp4 epsps*). Our lab has published data (Alexander et al., 2002; Sharma et al., 2004) demonstrating similarities in digestion of Roundup Ready® canola (event GT73), and the parental line from which it is derived, by ruminants, and in the behaviour of endogenous DNA from both lines. The focus of the present study, therefore, was on transgenic DNA only and specifically on quantifying the persistence of transgenic DNA in the rumen, at the proximal duodenum, and the feces of sheep fed forage-based and concentrate-based diets containing Roundup Ready canola meal. A second objective was to determine if transgenic DNA could be detected within microbial DNA or in blood.

5.2. MATERIALS AND METHODS

5.2.1. *Diets, animals and feeding*

Transgene stability was assessed using sheep fed forage-based (F) or concentrate-based (C) pelleted diets, both of which contained 15% (w/w; as-fed) canola meal (Table 5.1). Each diet was prepared using meal from genetically-modified (Roundup Ready®) canola and the non-GM parental line (event GT73) from which it was derived. The canola was provided by Monsanto Company (St. Louis, MO, USA), and the canola meals were prepared as described previously (Alexander et al., 2002). Samples of diets were ground to pass a 1.0-mm screen and dried at 135°C for 3 h (AOAC, 1990) for determination of ADF and NDF content (Van Soest et al. 1991; amylase and sodium sulphate included in the NDF procedure).

The study was conducted using six mature, ruminally and duodenally cannulated Canadian Arcott wethers, assigned randomly to diet groups F or C ($n = 3$). The wethers were penned individually, with free access to drinking water, and were fed at 0800 h daily. They were cared for in accordance with guidelines set by the Canadian Council on Animal Care (CCAC, 1993). Individual consumption of the pelleted diets was measured for the first 3 d of full adaptation, after which feeding was restricted to 95% of ad libitum intake.

The study comprised three feeding periods. In Period 1, the wethers were adapted over 14 d to the pelleted diets containing non-GM canola meal. For the next 11 d (Period 2), the diets containing R canola meal were fed. From d 12 onward (Period 3), feeding of the non-GM diets

was resumed. Each wether remained in its originally assigned diet group throughout the study.

5.2.2. *Sample collection*

Samples of ruminal fluid (RF), duodenal fluid (DF) and feces were collected as described by Alexander et al. (2004). Blood samples (5 mL per wether) were collected via jugular venipuncture. Except where specified, samples from each of the six wethers were processed individually.

On the last day of Period 1 (i.e., before any GM canola meal was fed), whole RF and DF were collected from one wether from each of diet group as a source of DNA for inclusion with the standards in real-time PCR assays and determination of the limit of detection, to simulate the in vivo matrices of the samples being assessed.

On days 3, 6 and 9 of Period 2, RF and DF samples were collected at 1200 h, pooled within diet groups, and duplicate 2-mL subsamples were clarified by centrifugation (13 000 × *g*; 10 min) to enable assessment of DNA isolated from cell-free supernatant. On days 4 and 7, RF and DF were collected at 1200 h and pooled within diet type. Subsamples from the composites were frozen for later extraction of DNA, and the remainders were used immediately in batch culture incubations as described below. On days 1, 5 and 9, blood samples were collected at 1300 h for extraction of DNA from 3-mL subsamples (not pooled). Persistence of transgenic DNA in the gastrointestinal tract was studied in unpooled digesta samples (RF, DF, feces) collected 1, 4, 7, 10, 13, 17, 21, 25, 29, 33, 37, 43 and 49 h after the last feeding of R diets (i.e., commencing at 0900 h on d 11 and continuing into Period 3 (d 12 and 13) when feeding of the non-GM diets had resumed).

5.2.3. *Batch culture incubations of ruminal and duodenal microorganisms*

The possibility of transformation of ruminal or duodenal microorganisms with transgenic DNA was investigated using an anaerobic batch culture incubation technique based on that described by Wang et al. (2000). Serum vials (35-mL capacity) were pre-loaded with 300 mg of ground, non-GM diets (F or C) as substrate and 15 mL of pre-warmed anaerobic buffer (Menke et al., 1979). To begin the incubation, 5 mL of whole RF or DF (pooled by diet F or C) were added to duplicate vials containing the corresponding substrate. The vials were sealed and affixed to a rotary shaker. After 18 h of incubation at 39°C, 5 mL of fermentation liquid were used as inoculum into fresh vials containing buffer and substrate (non-GM diets) only, and vials were incubated again for 18 h at 39°C. This process was repeated for a third 18-h subculture, after

which the entire contents of each serum vial were freeze-dried for subsequent assessment for transgene in extracted DNA. The serial subcultures were undertaken to ensure digestion of any GM plant material present in the original RF or DF inoculum (assumed to be complete after 54 h; Alexander et al. 2002), in order to preclude false positives in microbial DNA arising from contamination by recombinant plant DNA.

5.2.4. *DNA extraction and quantification*

A Wizard® Genomic DNA purification kit (Promega Corporation, Madison, WI) was used for extraction of DNA from blood samples according to the manufacturer's protocol. Isolation of DNA from cell-free RF and DF supernatants was performed as follows: 1 mL of the supernatant was extracted three times with an equal volume of chloroform/isoamyl alcohol (24:1), followed by precipitation in 0.6 volume of isopropyl alcohol, washing with 70% (v/v) ethanol, and resuspension in 50 µL of TE buffer. For extraction of DNA from freeze-dried whole RF, whole DF or fecal material, subsamples of approximately 1 g were ground to a fine powder using a planetary micro mill (Albisheim, Germany). In the case of batch cultures, the entire freeze-dried sample was ground in the same manner. The DNA from each powder was extracted using the CTAB method described by Lipp et al. (1999), with the following modifications: DNA was extracted from 180 mg of sample, and double volumes of each reagent were used at each step. Subsequently, the samples were incubated for 90 min in CTAB buffer for lysis and the final DNA was re-suspended in 200 µL of TE buffer. This same CTAB extraction method was also used to isolate DNA from Roundup Ready® canola meal for use as positive controls and real-time PCR standards. All DNA was quantified fluorometrically using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Burlington, ON) with a VersaFluor fluorometer (BioRad, Mississauga, ON).

5.2.5. *PCR analyses*

The primer sets used for PCR and the regions they amplified are described in Table 5.2. Primers for fragments F1, F2, and F3 were used to search for different sizes of transgenic DNA spanning the *cp4 epsps* construct in blood, digesta supernatants, and batch culture contents. The ovine-specific fragment GF was used as a positive control for amplification of DNA extracted from blood. The PCR conditions for F1 were: 95°C for 15 min; 40 cycles of 95°C for 20 s and 60°C for 1 min; 72°C for 10 min. Conditions for F2, F3 and GF were: 94°C for 15 min; 35 cycles

of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min; 72°C for 10 min. Fragments F4 and F5 were used to analyze transgenic DNA in whole RF and DF and feces. PCR conditions for F4 were: 94°C for 15 min; 74°C for 5 min; 35 cycles of 94°C for 1 min, 74°C for 3 min; 72°C for 10 min. Conditions for F5 were: 50°C for 2 min; 95°C for 10 min; 45 cycles of 95°C for 15 s and 60°C for 1 min. Universal primers were used as a positive control to detect bacterial DNA (fragment 16S; Table 5.2) encoding 16S from DNA extracted from RF and DF supernatants, feces, and batch cultures. Thermocycling conditions were: 94°C for 5 min; 30 cycles of 95°C for 15 s, 68.5°C for 30 s, and 72°C for 30 s; and an extension at 72°C for 10 min.

Each conventional PCR mixture (50 µL) contained (final concentrations): 1× HotStarTaq Master Mix (Qiagen Inc., Mississauga, ON, Canada), 0.15 µM of each primer, 200 ng template DNA, with the exception of positive controls for blood, in which case, 50 ng of DNA was added to each PCR. The PCR were performed with a DNA Engine Dyad (M.J. Research Inc., Watertown, MA, USA). Following each PCR, 20 µL of product was analyzed on a 1.5% (w/v) agarose gel.

Quantitative real-time PCR was performed using an iCyler iQ system (BioRad, Mississauga, ON, Canada) and primers amplifying fragment F5 (Table 5.2) for detection of transgenic DNA in whole RF, whole DF, and feces. Each PCR (50 µL) contained (final concentrations): 1× Universal Master Mix (Applied Biosystems, Foster City, CA, USA), 0.15 µM each primer, and 0.05 µM probe. The probe (Table 5.2) was labeled at the 5' end with the reporter dye 6-carboxyfluorescein (6-FAM) and at the 3' end with the quencher dye, 6-carboxytetramethylrodamine (TAMRA). Standards were prepared using DNA isolated from R canola meal, and concentrations of 1.0×10^5, 2.0×10^4, 1.0×10^4, 2.0×10^3, 1.0×10^3, 200, 100, and 50 pg per reaction. As the amounts of transgenic canola meal DNA standard were decreased, non-transgenic DNA was substituted in its place so that the final concentration of DNA (50 ng μL^{-1}) in each standard was kept constant. As well, DNA isolated from RF collected on the last day of Period 1 from one wether in each diet group, and pooled for extraction, was included with each standard. Each real-time PCR reaction contained 200 ng of DNA.

5.2.6. *Limits of detection of the 1363-bp* cp4 epsps *transgene*

Limit of detection assays were conducted to determine the sensitivity of the PCR for the 1363-bp *cp4 epsps* transgene (F4 from Table 5.2) in duodenal and ruminal matrices from wethers fed diet C or diet F. Four matrices were tested individually: 200 ng of extracted DNA from RF or DF sampled from a single animal fed either diet CP (animal 1) or FP (animal 4) on day 0 were

included in 50 µL PCR mixtures. Detection of the 1363-bp transgene was tested at concentrations of 10 000, 5 000, 1000, 500, 200, 100, 50, 25, 12.5, and 0 pg of purified R canola meal DNA per 50 µL reaction containing each matrix. Other PCR mixture components, thermocycling conditions and resolution of PCR products were identical to those described earlier for fragment F4.

5.2.7 Statistical analyses

The amounts of transgenic canola DNA measured by real-time PCR were analyzed by Proc Mixed using the covariance structure UN (SAS Institute, 1999). Differences were considered significant if the *P*-values were less than or equal to an α-value of 0.05.

5.3. RESULTS AND DISCUSSION

5.3.1. *Transgene detection and quantification in whole RF, DF, and feces*

Transgenic DNA was quantifiable by real-time PCR in DNA extracted from whole RF and DF for up to 21 h after the last feeding of F or C diets containing R canola meal (Figure 5.1). Beyond 21 h, most RF and DF samples analyzed resulted in transgenic DNA quantities less than the lowest standard (50 pg), which was considered the limit of quantification (LOQ).

None of the DNA extracted from fecal samples resulted in positive cycle thresholds (C_T), indicating the non-detectability of fragment F5 in feces. However, a 466-bp fragment of 16S DNA was amplifiable from each fecal sample (data not shown), suggesting that the results were not a result of PCR inhibition. This implies that transgenic DNA was substantially degraded between the duodenum and rectum, to levels at least as low as the limit of detection. This is supported by a previous study in which transgenes from GM maize and soybean were only detected in RF and DF of cattle, but not in feces (Phipps et al., 2003). It also reflects the form in which GM canola was fed to the wethers. Processing of canola seed to meal and pelleting of the meal in a mixed diet significantly increased digestion of the DNA associated with the meal, compared to whole canola seed, by exposing a greater amount of material available for digestion (Alexander et al., 2002). Passage of undigested whole canola seed to the rectum would likely result in detection of transgenic DNA.

Data from quantification of transgenic DNA in whole RF and DF from 1 to 21 h after feeding were analyzed statistically. Standard error was quite large as a result of variation among

individual wethers (Figure 5.1). Einspanier et al. (2004) were not able to reliably quantify transgenic DNA in the digestive tract of cattle fed GM maize silage. They did determine, however, a 97% decrease in transgene copy number as a result of ensiling, which highlights the importance of initial gene copy in the feed for quantitative results. Fragments of DNA up to 23 Kbp have previously been shown to be present in canola meal and total mixed diets containing canola meal (Alexander et al., 2002). Diet type (F vs. C) did not affect ($P = 0.19$) the quantity of transgenic DNA in RF or DF. However, there was a significant interaction between time of sampling and location of sampling within the digestive tract ($P = 0.04$; Figure 5.1). The maximum amount of transgenic DNA occurred at 7 h after feeding in RF (1240 pg). Thereafter, the decline was relatively steady, reaching a significant reduction at 17 h (588 pg; $P = 0.02$). In contrast, the concentration of transgenic DNA in DF was more variable. Transgenic DNA content was lower ($P = 0.04$) at 21 h (73 pg) than at 13 h (242 pg), however, differences in amounts of transgenic DNA among the other time points were not significant. The pattern of persistence of transgenic DNA in DF was similar to RF, but occurred later in the sampling period. This response likely reflects the transit time required for passage of canola meal from the rumen to the duodenum. At 1, 4, 10, 13, and 21 h after feeding, the concentration of transgenic DNA was greater ($P < 0.05$) in RF than in DF. The DNA quantified in DF ranged from 7% (at 1 h) to 35% (at 13 h) of that quantified in RF. Canola meal is a rapidly degraded protein supplement (62.3% DM disappearance after 12 h of incubation in the rumen; Piepenbrink and Schingoethe, 1998). While it does seem apparent that there is significant digestion of transgenic DNA within the rumen, it cannot be stated that 7 to 35% of transgenic DNA from canola meal passes from the rumen to the duodenum. This is because transgenic DNA was quantified within a fixed amount of total DNA (200 ng). The amount quantified therefore depends largely on other DNA, namely microbial DNA, extracted in the samples, which varies with time (Dehority, 2001) and location (Nicoletti et al., 1984) in the ruminant digestive tract.

Although transgenic DNA was quantifiable (>50 pg) up to 21 h after GM canola was withdrawn from the diet, in some cases it was detectable beyond that time, as indicated by a positive C_T during real-time PCR (Table 5.3). The 108-bp fragment F5 was detected up to 21 to 25 h in RF and 21 to 29 h in DF. The longer persistence in DF is probably due to passage time. At 25 h, the first meal of non-GM diets in Period 3 had been consumed, likely diluting any remaining transgenic canola meal, which in addition to its degradation and passage, rendered the transgene fragment undetectable shortly thereafter. We also investigated the detection of the 1363-bp *cp4 epsps* transgene (Table 5.3). This sequence of DNA was amplifiable from 1 to 13 h in RF, and from 4 to 13 h in DF. It has been theorized that transgenic plant DNA detected in a

ruminal environment is limited to that protected within intact plant cells (Alexander et al. 2002). If this were the case, one would expect that detection of one sequence of a plant genome would be equal to any other, irrespective of size. However, there were clear differences in persistence of F4 (1363 bp) compared to F5 (108 bp). Initially, we thought that this could be a reflection of the limit of detection (LOD) of F4, due to its size, in RF and DF. However, results from LOD assays utilizing DNA extracted from canola meal showed this not to be the case (Figure 5.2). Location-dependent differences in LOD were detected. The LOD was lower in matrices from the rumen (50 pg) compared to the duodenum (100 pg). These results emphasize the importance of considering matrix effects in the detection of transgenic DNA in digesta contents. However, even when the amount of transgenic DNA quantified were greater than the reported LOD (Figure 5.1), detection of F4 was not always possible. There is evidence that transgenic DNA is associated with solid plant material in RF (Sharma et al. 2004; Phipps et al., 2003), as was the case in this study (described below). Fragment F5 was not detected at any time in the aqueous phase of any digesta sample tested. It appears, therefore, that despite being associated with solid plant material in RF and DF, the detection of transgenic DNA may not necessarily reflect protection within plant cell walls. Differential digestion of plant DNA seems to occur within the feed residue, which may be related to the nature of the ruminal bacteria involved in the digestion of feed particles. Detection of transgenic DNA in digesta samples, therefore, is dependent on the selection of the fragment size to be amplified.

From previous *in vitro* studies, transgenic DNA in diets containing GM canola meal has been shown to be rapidly degraded in ruminal (Sharma et al., 2004) and duodenal cultures (Alexander et al., 2004). In RF batch cultures, transgenic DNA could not be detected beyond 8 h of incubation. Our results demonstrate that transgenic DNA was quantifiable for up to 21 h in RF and DF and detectable for 21 to 29 h (Table 5.3). However, this study does not imply that canola DNA is stable in the rumen for that length of time, as feed consumption was in some cases protracted. Feed remained in the bunks of some wethers up to 13 h after the morning delivery, despite being limited to 95% of ad libitum intake. The disparity between these studies therefore reflects differences between the closed (*in vitro*) system and the *in vivo* circumstance studied here, in which ingestion of feed into the rumen occurred over a 6- to 13- h period. Detection and quantification of transgenic DNA *in vivo* therefore depends on the quantity in feed (i.e., the proportion of GM plants in the diet), on feed intake, feed degradability, passage rates, and dilution by microbial or endogenous plant DNA.

5.3.2. *Transgene detection in digesta supernatants and blood*

Digesta and blood samples were collected during Period 2 (when F and C containing GM canola meal were fed) to investigate the fate of free transgenic DNA released from solid plant residue. Primers amplifying three fragments of DNA (F1, F2, and F3; Table 5.2) ranging in size from 62 to 420 bp and spanning the transgenic construct found in R canola were used. None of these fragments of transgenic DNA was detected in any of the RF or DF supernatant samples collected approximately 4 h after feeding on d 3, 6, or 9 (data not shown). In contrast, detection of transgenic DNA in whole RF and DF was possible for up to 21 or 29 h after the last feeding of the GM diets (Table 5.3). We tested for several small fragments because there is evidence that free DNA in the rumen is rapidly degraded (Flint and Thomson, 1991; Ruiz et al., 2000; Alexander et al., 2004). The results from this study suggest that the majority of transgenic canola meal DNA was associated with solid plant material in RF and DF. This has been supported previously by both *in vitro* and *in vivo* studies. Detection of sequences of transgenic DNA ranging from 179 to 1363 bp was limited to pelleted plant substrates throughout ruminal fluid batch cultures (Alexander et al., 2002; Sharma et al., 2004). Phipps et al. (2003) analyzed gene stability throughout the digestive tract of dairy cows fed insect-resistant corn and herbicide-tolerant soybean meal and could detect neither 203-bp nor 171-bp fragments of transgenic DNA from corn or soybean, respectively, in the liquid phases of RF or DF. However, those researchers did detect a 167-bp sequence of the high copy chloroplast *rubisco* gene, implying the importance of gene copy number in plant DNA detection.

It is likely that upon digestion of plant material, some free DNA is released into the aqueous digestive environment, as confirmed by detection of high copy plant DNA in the liquid phases of RF and DF (Phipps et al., 2003). Evidence from an *in vitro* study indicates that such is also the case for transgenic DNA (Alexander et al., 2004). In that report, however, detection of transgenic DNA was limited to small DNA fragments (62 bp) and the quantity of transgene in the DNA extracted from the feed residue was relatively high (approximately 2.5×10^4 to 3.5×10^4 copies per 245 ng of DNA). In the present study, the average amount of transgenic DNA extracted from whole RF or DF did not exceed 1240 pg or 367 pg, respectively, in 2.0×10^5 pg of extracted DNA. Any free transgenic DNA released into the rumen was probably rapidly eliminated or degraded to below detectable levels. The fact that transgenic DNA was not detected in the aqueous phase of RF from either diet would suggest that an abundance of microbial nucleases were present to rapidly degrade DNA in both concentrate- and forage-based diets. Negative results for transgenic DNA in DF supernatant was likely not a result of enzymatic degradation of DNA, however, as the pH of DF is too low for optimal activity of such enzymes

(Armstrong and Hutton, 1974). Instead, these results reinforce the instability of free plant DNA in the rumen and upper digestive tract of ruminants. All of the supernatant samples studied here did contain quantifiable DNA, most of which was likely microbial. A 466-bp fragment of 16S DNA was amplifiable from each sample indicating the presence of bacterial DNA. This therefore suggests that the inability to detect transgenic DNA in RF and DF supernatants did not result from PCR inhibitors.

The same fragments of DNA (F1, F2, and F3) sought for amplification in cell-free digesta supernatants were analyzed in DNA extracted from blood. In addition, blood was also analyzed for fragment F5 by real-time PCR. The ovine growth factor gene (positive control) was amplifiable in all of the blood samples tested, confirming that extracted DNA was of sufficient quality for PCR (Figure 5.3A). However, none of fragments F1, F2, or F3 was visible by gel electrophoresis (Figure 5.3B) after PCR of any of the DNA samples, nor did real-time PCR yield positive C_T for F5 (data not shown).

There is evidence that absorbed DNA may be associated with leukocytes (Schubert et al., 1997; Einspanier et al., 2001). Therefore, DNA was extracted from whole blood so as to extract DNA associated both with blood cells and plasma. In order to cross the intestinal barrier, transgenic DNA would likely have to exist in a free form in the fluid phase of digesta. The non-detectability of free transgenic DNA in the aqueous phase from DF suggests that fragments of free DNA would have had to be released at some point distal to the duodenum for absorption across the intestinal epithelium to occur. This study confirmed that transgenic DNA did reach the small intestine (Table 5.3), but it was degraded to below detectable levels, as fragments of the transgene were not detected in the feces. Whether or not transgenic DNA did exist in free form, separate from solid feed residue, between the duodenum and rectum, is unknown. Einspanier et al. (2001) also did not detect transgenic DNA in the blood of cattle given GM feed. In contrast, however, Mazza et al. (2005) did detect transgenic DNA in the blood of swine. This may have resulted from differences in the concentration of transgene reaching the intestine for absorption, as a consequence of differential intake (i.e., proportion of GM constituents in the feed) and digestion between the animals in these studies. The diets fed by Mazza et al. (2005) contained 50% (w/w) GM corn, compared with 15% (w/w) canola meal in the diets fed in the present study. Moreover, digestion of plant DNA prior to passage to the intestine may be greater in ruminants than in monogastric animals because of a longer retention time and more active microbial population in the digestive tract distal to the small intestine. As mentioned above, the average amount of transgenic DNA extracted from whole DF did not exceed 367 pg in 2.0×10^5 pg of total DNA. This substantial dilution of transgenic DNA in non-transgenic DNA would limit its

probability of absorption. Plant DNA does apparently cross the intestinal epithelium, as evidenced by fragments of endogenous high copy plant DNA detected in blood (Einspanier et al., 2001), muscle (Nemeth et al., 2004), and milk (Nemeth et al., 2004) of cattle. The extent of this uptake is likely dependent, however, on the number of copies of a gene found in feed, which itself is directly related to the gene copy number within the plant's genome and concentration of GM feed in the diet.

5.3.3. *Persistence of transgenic DNA in batch cultures*

It is difficult to determine, during periods when GM canola meal is being fed, whether or not bacteria in RF and/or DF are being transformed by transgenic DNA because of the confounding effect of contamination by the plant material itself. Thus, the decision was made to subculture *in vitro* the microbes in whole RF and DF collected during Period 2 (i.e., during intake of R canola meal) to test for stable incorporation of transgenic DNA. All of the RF and DF used as inoculum in the initial batch culture incubation tested positive for plant fragments F1, F2, and F3 and for bacterial fragment (16S; data not shown). After 54 h of subculturing (3 × 18 h), however, none of these transgenic plant DNA fragments was amplifiable in the batch culture incubation liquids, whereas fragment 16S (bacterial control) was still detectable (data not shown). This suggests that the transgenic DNA fragments detected in RF and DF were associated with suspended plant material, and not with DNA within the microbial genome. Alterations in mixed microbial populations in the gut are known to occur in response to changes in the diets fed to ruminants, e.g., feeding grain vs. hay (Tajima et al., 2001). Regardless of the diet type in the present study, however, stable incorporation of transgenic DNA into microbial genomes did not occur at detectable levels in batch cultures. The same is true for fragments F4 and F5 in the microflora of RF and DF, as shown by the finite intervals during which the fragments were detectable (Table 5.3). Low-copy transfer of transgenic plant DNA to gut microflora of humans has been described (Netherwood et al., 2004). In ruminants, naked plasmid DNA has been shown to lose the capacity to transform bacteria within 1 min of incubation in RF (Duggan et al., 2001), and the present study demonstrated non-detectability of free transgenic DNA from canola meal in cell-free RF and DF supernatants, implying that the instability of plant DNA in ruminant digesta is an important limitation to transforming events involving microbes associated with the fluid phases of ruminal and small intestinal digesta. Additionally, dilution of transgenic DNA likely reduces the probability of this process. At its peak concentrations in whole RF and DF, transgenic DNA accounted for only 0.54% (w/w) and 0.18% (w/w) of the total DNA,

respectively. This amount is even lower if one considers that the average transgenic construct in GM plants is only 2000-4000 bp of the entire genome. It remains to be determined if the microflora that are closely attached to feed particles are exposed to free plant DNA for an extended period of time.

In conclusion, the present study revealed that the disappearance from the rumen of transgenic DNA in diets containing 15% (w/w; as fed) Roundup Ready® canola meal results from ruminal digestion and passage to the small intestine. This was indicated by detection, in the duodenum, of genes as large as 1363 bp. However, transgenic DNA located in the duodenum is degraded to such an extent that it escapes detection by the time it passes to the rectum of sheep. The inability to detect free plant DNA in supernatant from ruminal and duodenal fluids is a limiting factor in transformation of microbes and absorption across the intestine. Evidence of either of these events occurring was not found. The dilution of transgenic DNA amongst other plant and microbial DNA is an additional hurdle to its uptake by bacteria or the animal. Quantitative real-time PCR can be used to investigate the fate of plant DNA in ruminants, but this may be limited by the starting quantity of DNA in the feed and its digestion. Quantification of genes specific to individual plants may allow for comparison of digestion in the rumen, given that the majority of plant DNA appears to be associated with solid feed residues. The manner in which it is associated, however, requires further study as it is not clear whether plant DNA is confined within plant cell walls or the solid feed matrix. Detection of transgenic DNA in livestock is dependent on a number of factors, including matrix effects, limits of detection, quantity of transgene in the feed, digestibility of the feed, and differences in the digestive physiology among livestock.

Table 5.1 Ingredients of experimental diets

Diet	FP	FR	CP	CR
Ingredients (kg t^{-1}, as fed)				
Alfalfa Meal	352.51	352.51	80.7	80.7
Barley (ground)	256.3	256.3	724.5	724.5
Parental Canola Meal	150	0	150	0
Roundup Ready® Canola Meal[a]	0	150	0	150
Beet Pulp	203.17	203.17	0	0
Calcium Carbonate	0	0	7	7
Sheep Mineral[b]	7	7	7	7
Vit ADE[c]	0.25	0.25	0.25	0.25
Dry Molasses	20	20	20	20
Maxi-Pel[d]	5	5	5	5
Canola Oil	5	5	5	5
Decox[e]	0.13	0.13	0.13	0.13
Chemical Analyses				
Dry matter	88.69	88.19	88.49	87.89
Organic Matter (%, DM basis)	91.28	93.73	92.15	93.88
NDF Content (%, DM basis)	38.55	34.34	28.07	23.47
ADF Content (%, DM basis)	23.57	19.19	13.49	10.83

[a] Roundup Ready canola is a registered trademark of Monsanto Technology LLC.

[b] Containing (%): NaCl (93.1), Mg (1.25), Zn (0.9), Mn (0.94), Cu (0.13), Se (0.003), K (1.25), Fe (1.25).

[c] Containing (IU g^{-1}): Vitamin A (10 000), Vitamin D (1250), Vitamin E (10)

[d] Feed pellet binder.

[e] Decoquinate (60 g kg^{-1}).

Table 5.2 Description of primers and a probe used in the amplification of Roundup Ready®-specific plant, bacterial, or ovine DNA

Fragment name	Primer or probe sequence (5' to 3')	Target
Plant specific		
F1[a] (62 bp)	(F[b]) CCA CGG CGT GCA TGC (R[d]) ACC AGA GGA CTT ACG AGC AGT TG	chloroplast transit peptide/*cp4 epsps*[c]
F2 (300 bp)	(F) CAA CAC TGG TAA GGC TAT GC (R) GGT AAC TGG AAG ACG ATC AC	Within *cp4 epsps*
F3 (420 bp)	(F) TTG ATT GCG ATG AAG GTG AG (R) ACA AAT GGT ACA AGA AAA ACA G	*cp4 epsps*/terminator
F4 (1363 bp)	(F) TCA CGG TGC AAG CAG CCG TCC AGC (R) TCA AGC AGC CTT AGT GTC GGA GAG TTC G	Entire *cp4 epsps* gene
F5 (108 bp)	(F) CCA TAT TGA CCA TCA TAC TCA TTG CT (R) GCT TAT ACG AAG GCA AGA AAA GGA (Pr[e]) TTC CCG GAC ATG AAG ATC ATC CTC CTT	3' insert-to-plant junction
Bacterial Specific		
16S (466 bp)	(F) TCC TAC GGG AGG CAG CAG T (R) GGA CTA CCA GGG TAT CTA ATC CTG TT	16S rDNA
Ovine Specific		
GF (652 bp)	(F) CAA CAG GAAGGA ATC ATT ACA GTA (R) CCA AAA CAG CCG CTT ATC CAA G	Growth factor

[a] Fragment F1 from Alexander et al. (2004); F2 and F3 from Sharma et al. (2004); F4 from Alexander et al. (2002); F5 from Monsanto (2005); 16S from Nadkarni et al. (2002); GF from Lanneluc et al. (1996).

[b] Forward primer.

[c] Transgene encoding the synthetic enzyme 5-enolpyruvylshikimate-3-phosphate synthase derived from *Agrobacterium sp.* strain CP4.

[d] Reverse primer.

[e] Probe for real-time PCR.

Table 5.3 PCR detection[a] of transgenic DNA in whole ruminal and duodenal contents of sheep fed diets containing 15% (w/w; as fed) Roundup Ready® canola meal

	Diet											
	Concentrate- based						Forage- based					
	Sheep Number											
Time (h)	1		2		3		4		5		6	
	RF	DF	RF	DF	RF	DF	RF	DF	RF	DF	RF	DF
1[b]	e w	e -	e w	e -	e w	e -	e w	e -	e w	e -	e w	e -
4	e w	e w	e w	e -	e w	e -	e w	e -	e -	e -	e w	e -
7	e w	e -	e w	e -	e w	e w	e w	e w	e w	e w	e w	e w
10	e w	e -	e -	e -	e w	e -	e w	e -	e w	e w	e -	e -
13	e -	e -	e -	e -	e -	e -	e w	e -	e -	e -	e w	e -
17	e -	e -	e -	e -	e -	e -	e -	e -	e -	e -	e -	e -
21	e -	e -	e -	e -	e -	e -	e -	e -	e -	e -	e -	e -
25	- -	e -	- -	- -	e -	- -	e -	e -	- -	- -	- -	e -
29	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	e -
33	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
37	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
43	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
49	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -

[a] Detection of a 108 bp fragment spanning the 3' insert-to-plant junction region (e) by real-time PCR or a 1363 bp fragment spanning the whole *cp4 epsps* gene (w) by conventional PCR. The fragments were considered positive when PCR resulted in a positive cycle threshold (e) for the 108 bp fragment or the proper band size when visualized by gel electrophoresis (w) for the 1363 bp fragment. Negative detection (-).

[b] Time point zero hour was considered to be 0800 h on day 11 of the trial when animals were fed diets containing Roundup Ready® canola meal. On day 12, the animals were fed diets containing parental canola meal (24 h), as described in section 5.2.1.

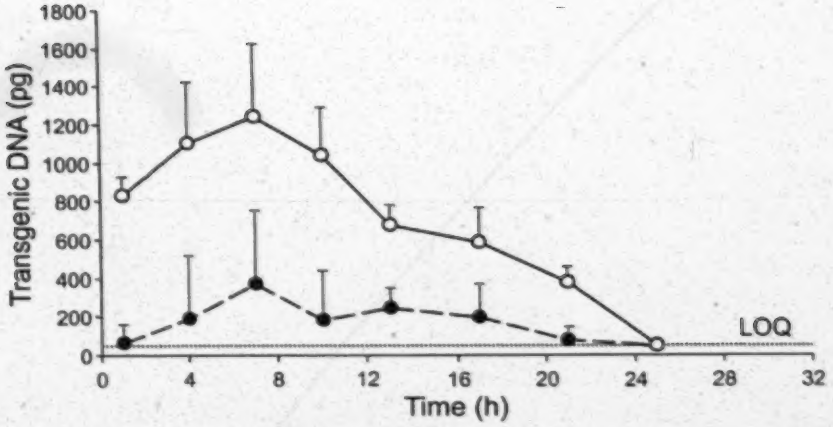

Figure 5.1 Quantification of transgenic DNA in whole ruminal fluid (open circles) and duodenal fluid (closed circles) from sheep fed diets containing 15% (w/w; as fed) Roundup Ready® canola meal. At 0 h, animals were fed diets containing transgenic canola meal for the last time. At 24 h, animals were switched to diets containing 15% (w/w; as fed) parental canola meal. The limit of quantification (LOQ) was considered to be 50 pg. Statistical analysis was only performed on time points 1 to 21 h.

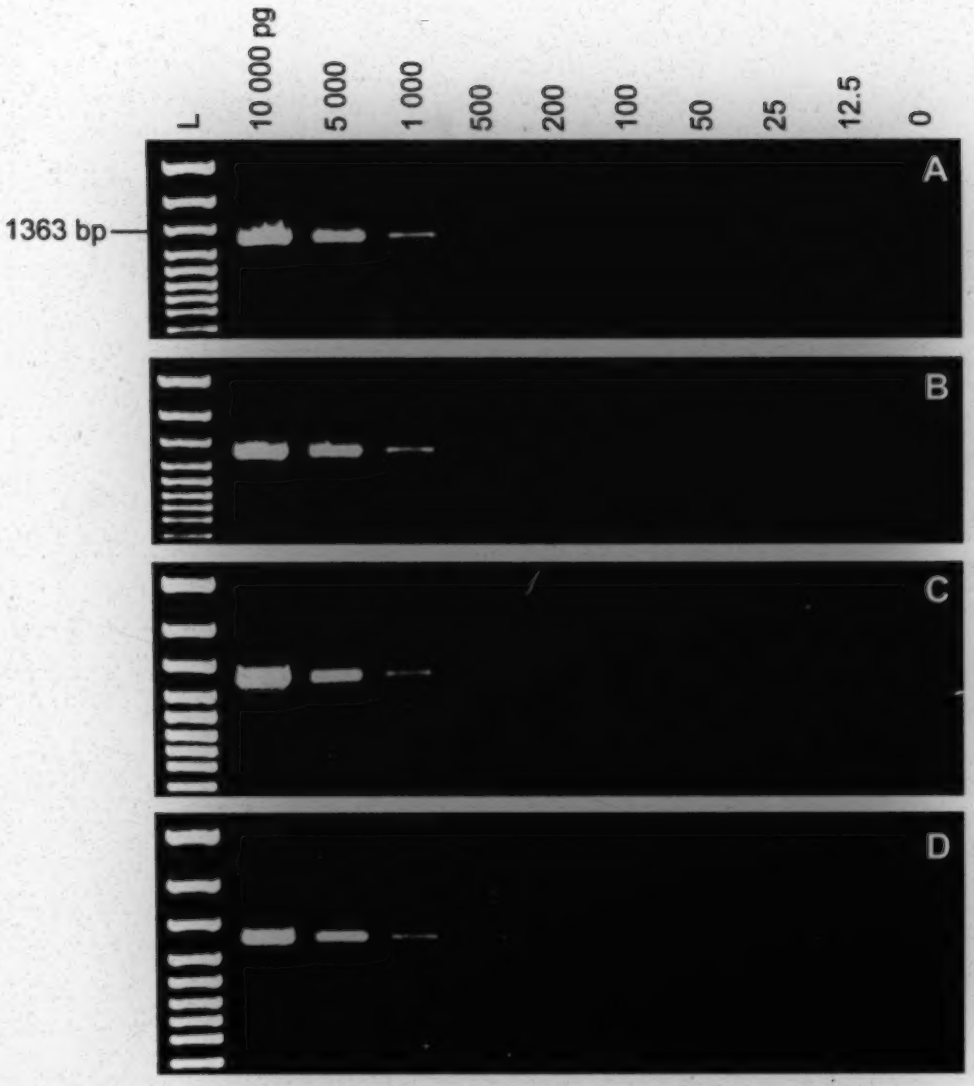

Figure 5.2 PCR limits of detection of the 1363 bp *cp4 epsps* transgene in DNA extracted from ruminal (A, C) and duodenal (B, D) digesta of wethers fed a concentrate- (A, B) or a forage- (C, D) based diet. All PCRs contained 200 ng of matrix DNA from ruminal or duodenal digesta in addition to 10 000, 5 000, 1 000, 500, 200, 100, 50, 25, 12.5, or 0 pg of extracted canola meal DNA. L; DNA ladder.

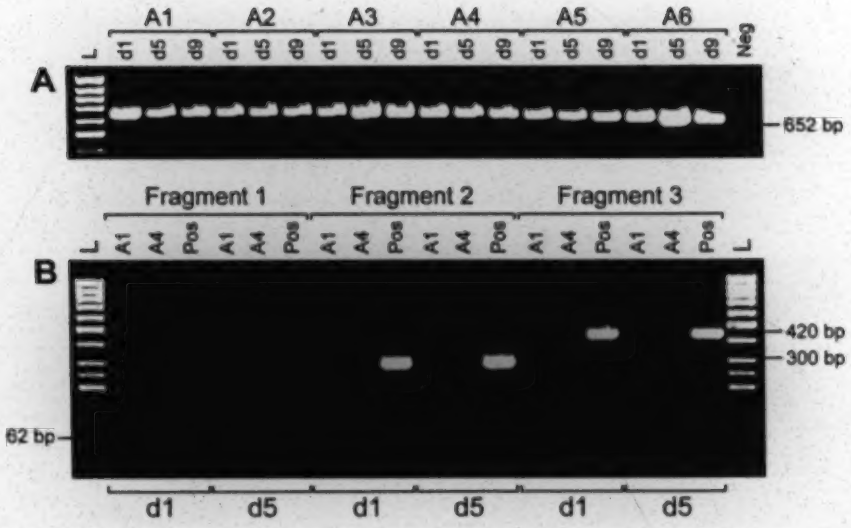

Figure 5.3 Gene detection in DNA extracted from whole wether blood while being fed diets containing 15% (w/w; as fed) GM canola meal. Animals (A) 1, 2, and 3 were fed a concentrate-based diet and A4, 5, and 6 were fed a forage-based diet. A. Detection of an ovine specific growth factor gene in blood on days (d) 1, 5, and 9. L; DNA ladder. Neg; negative control containing all components of the PCR except template DNA. B. Representative analyses for PCR of transgenic fragments in blood from A1 and A4 on d1 and 5. All blood samples tested negative for 62 bp, 300 bp, and 420 bp fragments of transgenic DNA. L; DNA ladder. Pos; positive control containing DNA extracted from Roundup Ready® canola meal.

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Chapter 6 – Use of Quantitative PCR to Predict Dry Matter Disappearance of Individual Feeds in a Total Mixed Diet

6.1. INTRODUCTION

The current feeding methods for ruminants are based on net energy or metabolizable energy systems (Lopez et al., 2000). Both systems are dependent on the digestibility of feeds, which can account for most of the variation in predicting available energy for production parameters (Minson, 1990). Each system is limited by using a constant energy value for a single feed whether fed alone or in combination with other feeds (Lopez et al., 2000). None of the systems incorporate associative effects between feeds which are known to occur in mixed diets (Mould et al., 1983; McDonnell, et al., 1979).

Associative effects, whether positive or negative, can result in deviation between the actual and predicted digestibility of mixed rations (Dixon and Stockdale, 1999). For example, negative associative effects resulting from the addition of grain to forage result from a reduction in rumen pH due to the rapid digestion of fermentable carbohydrates in grain. Microbial digestion of fibre components in forages is optimal at a pH range of 6.6-7.0 (Terry et al., 1969; Hiltner and Dehority, 1983). In addition, changes in the microbial populations resulting from lowering of the pH as a result of inclusion of readily fermentable starch may attribute to the negative associative effects (Mould et al., 1983). Therefore, the energy value of a feed component can change when mixed with other feeds.

Currently, there is no method available to directly quantify the digestion of individual feed components in a mixed diet. Such a method could contribute significantly to predicting the available energy of a mixed diet depending on the concentration and the nature of the feed components that are included in the diet. The ability to measure digestion of each dietary component would be dependent on quantifying a marker unique to each feed substrate or plant. There is evidence that in the rumen, low-copy plant genes are associated only with feed residues and that a relationship between plant cell degradation and the persistence of DNA exists (Phipps et al., 2003; Alexander et al., 2002). Thus unique plant DNA markers may provide the means to estimate the digestibility of specific plant cells within a mixed diet. The objectives of this work were to establish regressions between DNA and DM disappearance for mixed diets consisting of corn and alfalfa incubated in ruminal fluid. From this, DM digestibility of corn and alfalfa in diets containing varying concentrations of each were estimated.

6.2. MATERIALS AND METHODS

6.2.1 *Ruminal Fermentation substrates*

Fresh alfalfa forage was dried at 55°C for 48h and then ground to pass through a 1-mm screen. Whole corn seed was ground to pass through an 8-mm screen. Dry matter (DM) content of alfalfa and corn were determined after incubation for 24h at 105°C. For the ruminal fermentation experiment the substrates were 100% alfalfa (w/w; A100), 75% alfalfa plus 25% corn (w/w; A75:C25), 50% alfalfa plus 50% corn (w/w; A50:C50), 25% alfalfa plus 75% corn (w/w; A25:C75), and 100% corn (w/w; C100). Substrates were weighed (0.5 g DM) into filter bags (#57, ANKOM Corp., Fairport, NY).

6.2.2. *Ruminal fermentation*

The Daisy^{II} Incubator *in vitro* fermentation system was used to test the DM and DNA disappearance of each substrate, according to the manufacturer's instructions (ANKOM Corp., 2005). Two buffer mixtures of pH 6.8 or pH 5.5 were prepared. To achieve the lower pH of 5.5, less of the recommended amount of Buffer Solution B was added to Buffer Solution A. However, both of the pH 6.8 and 5.5 buffers were formulated to contain equal amounts of reducing agent ($Na_2S \cdot 9H_2O$; approximately 0.17 g L^{-1}). Rumen fluid (RF) was collected from a ruminally fistulated Angus steer maintained on a ground barley and barley silage diet and processed under anaerobic conditions. Processed RF inoculant (400 mL) was added to ANKOM fermentation jars containing either pH 6.8 or pH 5.5 buffers (1600 mL). Filter bags containing the substrates and empty reference filter bags were added to the fermentation jars and incubated at 39°C for 48h. Substrate bags were removed in triplicate (plus one empty bag per time point) at 0, 8, 16, 24, and 48 h of incubation, rinsed under cold water and then frozen in liquid nitrogen. The filter bags were freeze- dried and weighed to determine DM disappearance.

6.2.3. *DNA extraction and quantification*

The substrates were removed from the bags and ground to a fine powder using a planetary micro mill (Albisheim, Germany). DNA was extracted from each powder according to the CTAB method described by Lipp et al. (1999), with the following modifications; DNA was extracted from 170 mg of sample and two times the volume of each reagent was used at each

step. Subsequently, the samples were incubated for 90 min in CTAB buffer for lysis and the final DNA was re-suspended in 200 µL of TE buffer. The same CTAB extraction method was used to isolate DNA from canola meal, which was added to the real-time PCR standards as background DNA. All DNA was quantified fluorometrically using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Burlingtom, ON) with a VersaFluor fluorometer (BioRad, Mississauga, ON).

6.2.4. *PCR analyses and DNA disappearance*

Primers and probes for real-time PCR were designed using Beacon Designer 4.0 software (PREMIER Biosoft Int., Palo Alto, CA). Alfalfa DNA was quantified using forward primer AF (5'-TCG ACT ATT GTG GAG AAC CTT AGG-3'), reverse primer AR (5'-CAA TGT GGT CTG AGT TGA TTC GG-3'), and probe AP (5'-CGA CAA CAA CCC AAG CCC CGC CTC-3'), specific to the *Medicago sativa acetyl-CoA carboxylase* gene (Accession No. L25042). Corn DNA was quantified using forward primer CF (5'-CAC TCT CAG CTA CTT TCC TTC TCC-3'), reverse primer CR (5'-TGA ACT CAG CGT CCT TAT GTG G-3'), and probe CP (5'-ACA CCC ACC AGC ACA GCA ACA CCC-3'), specific to the *maize alcohol dehydrogenase 1* gene (Accession No. X04050). The probes were labelled at the 5' end with the reporter dye 6-carboxyfluorescein (6-FAM) and at the 3' end with the quencher dye 6-carboxytetramethylrodamine (TAMRA). An iCyler iQ system (BioRad, Mississauga, ON) was used for PCR. Each PCR (50 µL) contained (final concentrations): 1 x Universal Master Mix (Applied Biosystems, Foster City, CA, USA), 0.15 µM each primer, and 0.05 µM probe. The standards for alfalfa and corn real-time PCRs included 2.0×10^5, 1.0×10^5, 2.0×10^4, 1.0×10^4, 2.0×10^3, 1.0×10^3, 200, and 100 pg of alfalfa or corn DNA, respectively. As alfalfa or corn DNA was diluted, DNA from canola meal was substituted in its place so that the final concentration of DNA (50 ng μL^{-1}) in each standard did not change. For all PCR, each reaction contained 200 ng of DNA.

The amount of alfalfa or corn DNA quantified in 200 ng of extracted DNA was used to calculate the total amount of each type of DNA remaining at every time point for individual substrates throughout fermentation. These data were used to determine DNA disappearance for alfalfa and corn substrates.

6.2.5. *Statistical analyses*

Regression between DNA (independent variable) and DM (dependent variable) disappearance for the substrates containing 100% (w/w) alfalfa or corn (A100 and C100, respectively) were analyzed at pH 6.8 and pH 5.5 using Proc Nlin (SAS Institute, 1999). Correlations between DNA and DM disappearance for A100 and C100 were calculated using Proc Corr. The models derived from regression were used to predict the DM disappearance of alfalfa and corn in substrates containing mixed amounts of each (A75:C25, A50:C50, and A25:C75). The amount of predicted alfalfa and corn DM disappearance were summed together (total predicted DM disappearance) and compared against actual total DM disappearance to analyse the accuracy of the models by using the concordance correlation (Lin, 1989). The amount of predicted alfalfa and corn DM disappearance in each of the mixed substrates were compared against each other and against the actual DM disappearance of diets A100 or C100 using Proc Mixed with UN structure. Differences were considered significant if the *P*-values were less than or equal to an α value of 0.05.

6.3. RESULTS AND DISCUSSION

6.3.1. *Regression analyses*

The relationships between DNA and DM degradation for the control diets containing either 100% alfalfa or corn are shown in Figure 6.1. For alfalfa, a single model did not provide the most accurate predictions for DM disappearance at time points 8, 16 and 24 h in mixed diets (see below). Instead, the best model for prediction of DM disappearance occurred by formulating a linear model from time points 8 and 16 h (for predicting DM disappearance at 8 h) and a quadratic model from time points 16 to 48 h (for predicting DM disappearance at times 16 and 24 h). The regression models for alfalfa digested at pH 6.8 (linear: $y = 1.0573x - 68.775$; quadratic: $y = 2.929x^2 - 573.19x + 28075$) and pH 5.5 (linear: $y = 0.3813x - 10.379$; quadratic: $y = 0.6117x^2 - 118.07x + 5723.9$) differed however the trends were similar. The majority of DNA was degraded in the first 8 h at both pH's (88.2%, pH 6.8 versus 86.9%, pH 5.5). Despite this, significant amounts of alfalfa DNA were quantified at 48 h for each pH-treatment (57.8 µg, pH 6.8 versus 166.1 µg, pH 5.5; data no shown). Disappearance of DM and DNA in the first 8 h was likely a reflection of microbial digestion of parts of alfalfa that have a greater cell density and are more easily accessed, such as leaves. In legumes, the blades contain a greater proportion of thin-wall cells compared to the stem (Wilson, 1991) and it has previously been shown that digestion of alfalfa leaves is more rapid compared to stems (Albrecht et al., 1987). The slower

rate of disappearance of DM and DNA in the later time points probably represented digestion of plant material containing higher fibre content such as stem. When alfalfa is at the mid-flowering stage of maturity, approximately 25% of the leaf blade mass is neutral detergent fibre compared to 40-55% neutral detergent fibre content in the stems (Buxton et al., 1995). The excess fibre in the stems likely limited microbial access to plant cellular material and resulted in slower DNA disappearance seen in the later hours of digestion (Figure 6.1). The differences in digestibility of stems and leaves is likely why dividing the DNA and DM regression into two equations resulted in more accurate DM disappearance predictions (see below). This is further supported by regression analyses between DNA and DM disappearance. When including data from time points 8, 16, 24 and 48h, the correlation (r) between DNA and DM disappearance was 0.81 and 0.88 at pH 6.8 and pH5.5, respectively. However, when excluding the 8 h data, the correlation increased to 0.95 and 0.92 for pH 6.8 and pH 5.5, respectively.

For corn at pH 6.8, the best fit model was linear ($y = 1.3932x - 70.037$). Corn DM digestion has previously been shown to occur in a linear fashion (McAllister et al., 1990), which appears to be true for corn DNA at an optimal pH (Figure 6.1). The correlation between corn DNA and DM disappearance was 0.84 over 48 h. However, the relationship between DNA and DM disappearance was less predictable when corn was digested at pH 5.5 ($r = 0.59$). The model used for these data was quadratic ($y = 0.0445x^2 - 3.9779x + 96.485$). In the first 16 h, it appeared that DNA degradation occurred at a greater extent compared to DM (Figure 6.1). While the reason for this is not clear, it may be related to differential digestion of the embryo compared to the endosperm. It has previously been shown that the majority of DNA in the maize kernel is distributed almost equally between the endosperm and embryo, despite the embryo only accounting for 10-12% of the seed weight compared to the endosperm which can represent 80-85% of the weight (Trifa, 2004). Both DM and DNA disappearance were significantly less for corn at pH 5.5 compared to pH 6.8. Lower DM disappearance likely resulted from less endosperm cell wall digestion and thus starch granule digestion. Even though starch digestion occurs at pH 5.5 (Huntington, 1997) plant cell wall digestion, which is inhibited below pH 6.2 (Hiltner and Dehority, 1983), must first take place. Digestion of cell wall and cell contents would also be expected to decrease in the embryo. However, due to the high concentration of DNA in this part of the seed, even a low extent of DM disappearance would be expected to result in significant DNA disappearance. Perhaps a greater number of time points would provide better insight into DM and DNA disappearance of corn incubated in RF at pH 5.5.

6.3.2. *Estimation of total DM disappearance*

The formulas described in *Section 6.3.1* were used to estimate DM disappearance of alfalfa and corn in diets A75:C25, A50:C50, and A25:C75 (see below). To test the effectiveness of the models, the predicted DM disappearances were used to estimate the amount of alfalfa, corn, and total (alfalfa plus corn) DM remaining at each time point (data not shown). Theoretically, the summed amount of predicted DM for alfalfa and corn in each diet should equal the actual total DM. The predicted total DM disappearances compared against the actual DM disappearances for each mixed diet are shown in Figure 6.2. Differences in these values ranged between -9.78 and 5.71% and -15.18 and 12.12% for pH 6.8 and pH 5.5 treatments, respectively. The accuracy, or correctness, of the fitted models over time were tested by analyzing the concordance correlation coefficient (Lin, 1989) between the summed predicted and actual total DM disappearances for all mixed diets. The concordance correlation was 0.87, indicating that the models worked well in predicting DM disappearance from DNA disappearance. Differences between the actual and predicted total DM disappearances appeared to be slightly better for the pH 6.8 data compared to the pH 5.5 data. These differences were probably due to the less predictable regression for corn at pH 5.5. Despite this, it was evident that quantifying specific plant DNA by real-time PCR could be applied to estimate the DM disappearance of each substrate in the mixed diets.

6.3.3. *Disappearance of alfalfa and corn DM*

The predicted DM disappearances for alfalfa and corn in each of the mixed diets are shown in Figure 6.3. Only time significantly affected the DM disappearance of corn in the mixed diets. The same was true when comparing the actual DM disappearances of diet C100 at pH 6.8 and pH 5.5 (Figure 6.4). Bacteria that digest non-structural carbohydrates have been shown to persist in rumen fluid until the pH decreases to between 4.6 and 4.9 (Russell et al., 1979; Russell and Dombrowski, 1980). These findings support this study, in which the microbial populations digesting corn did not appear to be diminished at pH 5.5.

The discrepancy in DM disappearance predicted from DNA disappearance for the corn pH 5.5 data was evident, as corn DM disappearance decreased at 16 h for both diets A75:C25 and A50:C50. However, the predicted DM rate of degradation of corn in diet A25:C75 appeared to mimic the actual disappearance of 100% corn at pH 5.5 (Figure 6.4) implying that the model might fit better with increasing concentrations of corn in the diet.

Due to the variability from the pH 5.5 corn data, statistical analysis of only the corn DM disappearance at pH 6.8 was additionally performed. Even when excluding the pH 5.5 data however, DM disappearance was still only affected by time ($P < 0.05$). For each time point, the predicted DM disappearance did not differ across any of the mixed diets or the actual DM disappearance for diet C100. This suggested that even in the presence of increasing concentrations of alfalfa, and the microflora responsible for digesting alfalfa, corn digestion was not altered.

For alfalfa, DM disappearance was significantly affected by an interaction between time and pH. At both pH treatments, predicted alfalfa DM disappearance of the mixed diets (Figure 6.3) and the actual disappearance for diet A100 (Figure 6.4) increased over time. Disappearance of DM was greater at time 16 h and 24 h for the pH 6.8 treatment compared to pH 5.5. However, at 8 h, there was no difference in DM disappearance between treatments pH 6.8 or 5.5 ($P = 0.65$). This was likely the result of differences in digestibility of components within alfalfa that were mentioned in the above section. It is probable that up to 8 h of fermentation, components containing less fibre and that were more easily accessed by bacteria, such as leaf material, were digested to the same extent at pH 6.8 and pH 5.5. At 16 h and 24 h however, the majority of alfalfa substrate remaining probably consisted of less digestible components with higher fibre content, such as stems (Wilson, 1991). Populations of cellulolytic bacteria have been shown to diminish when the pH is less than 6.2 (Russell and Dombrowski, 1980; Russell et al., 1979) and fibre digestion is significantly depressed when the pH falls below 6.0 (Hoover, 1986).

An interaction between pH and diet additionally affected DM disappearance of alfalfa ($P < 0.05$). Each of the predicted DM disappearances for the mixed diets (A75:C25, A50:C50, and A25:C75) and the actual DM disappearance of diet A100 were greater at pH 6.8 compared to pH 5.5. Within the pH 5.5 treatment, DM disappearance was not different between any of the diets ($P > 0.05$), indicating that at pH 5.5, alfalfa was digested to the same extent, regardless of its concentration in the diets. At pH 6.8, alfalfa DM disappearance was not different between diets A100, A75:C25, and A50:C50 ($P > 0.05$). However, DM disappearance of diet A25:C75 was significantly different from A100 and A50:C50 ($P < 0.05$) and tended to be different from A75:C25 ($P = 0.09$). Negative associative effects due to the addition of readily fermentable carbohydrates (RFC) to forage diets have previously been shown *in vitro* (Mertens and Loften, 1980; Hiltner and Dehority, 1983) and *in vivo* (Joanning et al., 1981; Miller and Muntifering, 1985). Reduction in ruminal fibre digestion has been suggested to arise from several factors including a decrease in pH due to the rapid fermentation of RFC (Hoover, 1986). It was evident in this study that a pH of 5.5 decreased DM disappearance in the fibrous portion of alfalfa.

Additionally, reduced fibre digestion due to the addition of RFC may result from changes in microbial populations and a preference by microbes for RFC compared to the more complex-structured cellulose (Mould et al. 1983). Mould et al. (1983) showed that decreased fibre digestion in a mixed concentrate and hay diet could not completely be alleviated when the pH was maintained at 6.7. In this study, DM disappearance of alfalfa was different for the mixed diet containing the highest concentration of corn (A25:C75), compared to the other diets within the pH 6.8 treatment. However, unlike the study by Mould et al. (1983), changes in microbial populations resulting from the inclusion of RFC did not appear to lower digestion of alfalfa. Instead, it appeared that alfalfa DM disappearance may have been improved at 8 h and 16 h for diet A25:C75 (Figure 6.3). The differences in this study and that by Mould et al. (1983) may reflect the type of RFC used. In the present study, corn was added to alfalfa whereas Mould et al. (1983) added barley to hay. Barley has previously been shown to have a greater rate of digestion compared to corn, due to structures of corn that are resistant to digestion (McAllister et al., 1990). This may explain why there was not a shift away from alfalfa digestion in our study. It could be that corn was not a more fermentable substrate compared to alfalfa at 8 h and 16 h for the pH 6.8 treatment. If there was a shift towards corn digestion, it might be expected that DM disappearance of corn in diet A25:C75 would have been greater than the other diets. This showed not to be the case, as only time affected corn DM disappearance ($P < 0.05$).

From the results of these experiments, it appeared that DM disappearance of alfalfa and corn in mixed diets was affected by the general environment (pH) and the microenvironment of microflora created by different concentrations of alfalfa and corn in the mixed diets. The effects of these factors can result in negative or positive associative effects. This is supported by previous studies (Mould et al.,1983; Hoover, 1986). The current systems to predict net energy or metabolizable energy in mixed diets do not take into account associative effects caused by the interaction of one feed and another (Lopez, 2000). Measuring associative effects is limited by the inability to directly estimate disappearance of individual feeds in mixed diets. Components common to each feed, such as fibre or DM, are measured and it is not known to what extent individual feeds are digested. The application of real-time PCR may provide further insight into how mixtures of feeds affect overall digestibility and individual feed digestibility.

For *in vivo* studies, prediction of DM digestion of a test feed mixed with a second feed has been done by comparing the difference between digestion of only the test feed and the test feed in combination with the second feed (Rymer, 2000). This method does not consider associative effects and is limited when the test feed cannot be fed at high concentrations, such as the case of protein supplements. In such instances, regressions have been used to extrapolate data

in order to predict the digestibility of 100% supplement, without considering associative effects. Real-time PCR has the potential to consider associative effects when comparing digestibility of mixed feeds. In addition, real-time PCR may be useful to predict digestibility of feeds that cannot be included in diets at high concentrations, such as protein supplements. The disappearance of canola meal DNA has previously been shown to correlate well to DM disappearance (Alexander et al., 2004). There may be limitations to utilizing real-time PCR for digestion studies however. Plant DNA may be degraded to an extent that is not quantifiable or it may be heavily diluted in microbial DNA, also limiting quantification. In these cases, the method would overestimate DM disappearance, as not all fibre is completely degraded. Using higher-copy genes for quantification may help reduce this error. High-copy plant genes have been detected in the feces of cattle when low-copy plant genes could not be detected (Phipps et al., 2003). Further studies to test the usefulness of real-time PCR in predicting DM disappearance of single components in mixed diets *in vitro* and *in vivo* are warranted. This method offers the potential to independently compare the digestion of feed components throughout the digestive tract of ruminants.

In conclusion, this study showed that real-time PCR can be applied to determine the DM disappearance of both alfalfa and corn in mixed diets containing varying concentrations of each. Previously, there has been no method to predict DM disappearance of single feed components in a mixed diet without excluding associative effects. It appeared that both the larger external environment, such as pH, and the microenvironment created by mixed corn and alfalfa feeds, can lead to associative effects altering DM disappearance. Further studies are warranted but for the first time, a method has been proposed to measure digestion of each feed ingredient in a mixed diet, while considering associative effects. By quantifying digestion of individual feed components in a mixed diet, a better understanding of digestive kinetics may be achieved. This may lead to improved and more economical diet formulations.

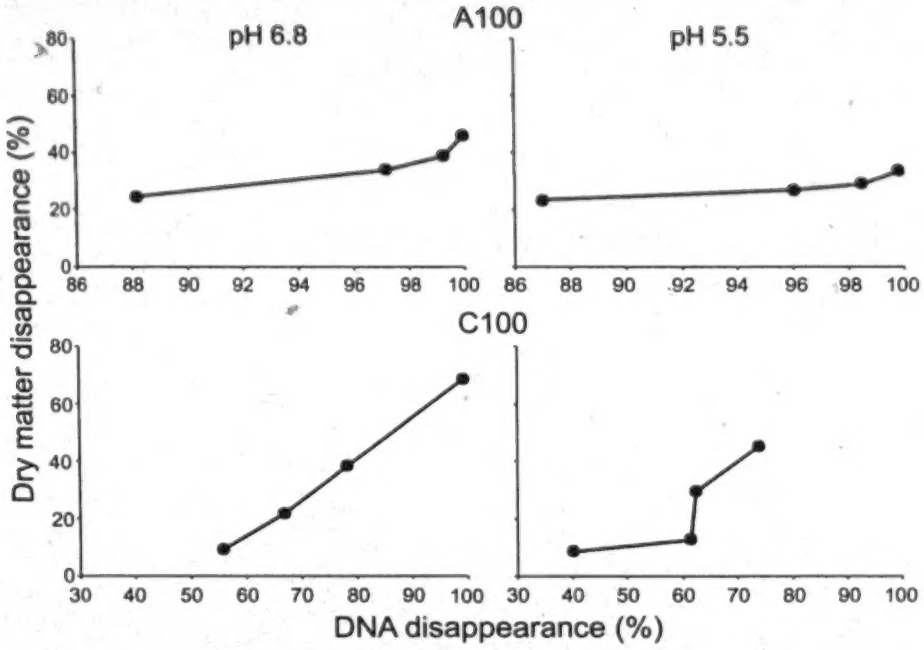

Figure 6.1 Regression analysis between DNA and dry matter disappearance of 100 percent alfalfa (A100) or corn (C100) incubated in rumen fluid at pH 6.8 or pH 5.5.

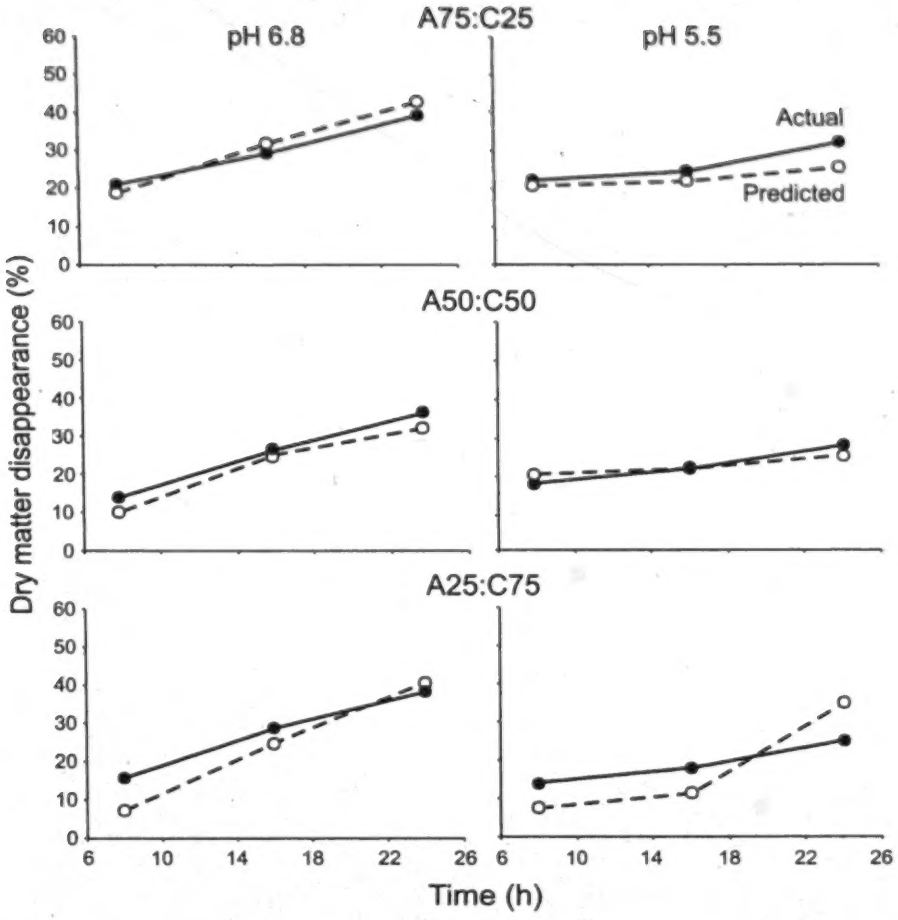

Figure 6.2 Plotting of the predicted versus actual total dry matter disappearance of mixed diets containing 75:25 (A75:C25), 50:50 (A50:C50), and 25:75 (A25:C75) percent alfalfa:corn in rumen fluid at pH 6.8 or pH 5.5. Predicted dry matter was estimated by summing the predicted dry matter of alfalfa and corn at each time point for each diet.

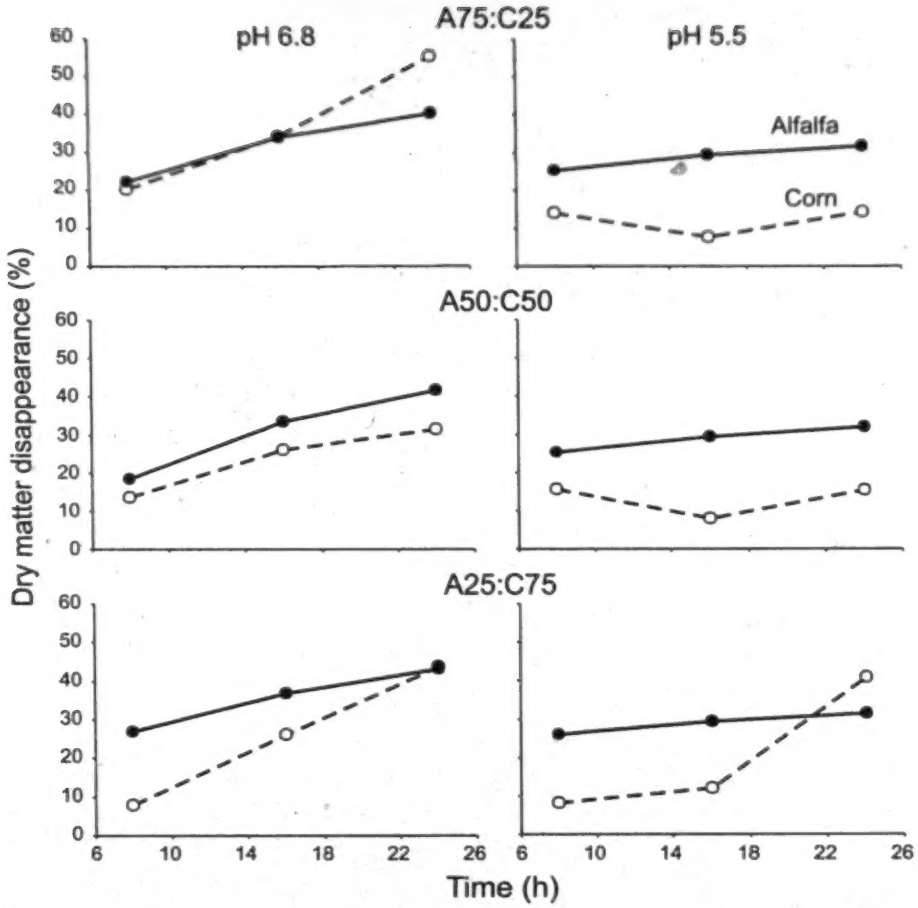

Figure 6.3 Estimated dry matter disappearance of either alfalfa or corn in mixed diets containing 75:25 (A75:C25), 50:50 (A50:C50), and 25:75 (A25:C75) percent alfalfa:corn in rumen fluid at pH 6.8 or pH 5.5.

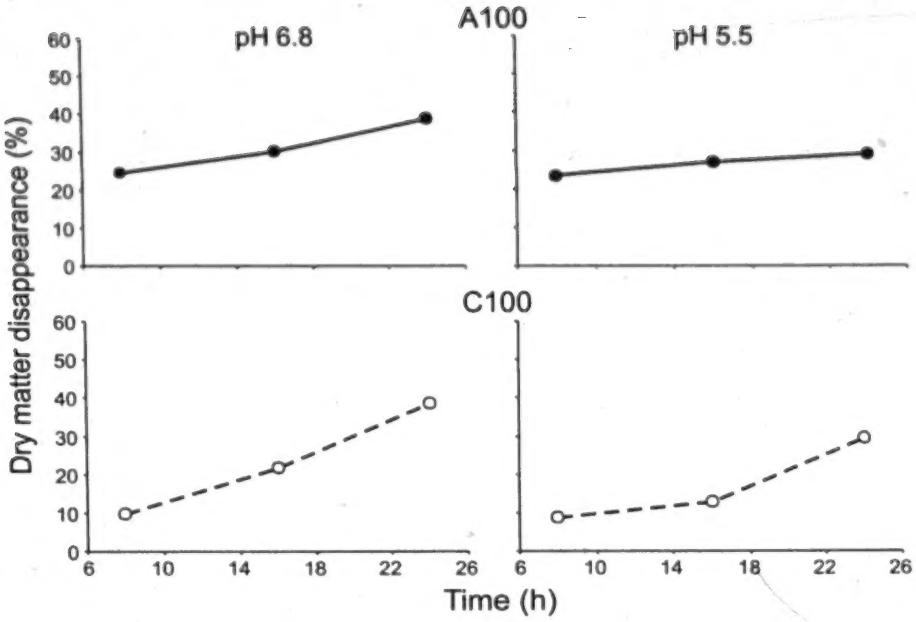

Figure 6.4 Actual dry matter disappearance of 100 percent alfalfa (A100)or corn (C100) incubated in rumen fluid at pH 6.8 or pH 5.5.

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Chapter 7 – General Discussion and Conclusions

Since the commercialization of genetically-modified (GM) crops, there have been concerns at public and scientific levels regarding the fate of transgenic DNA in GM crops fed to livestock (Gasson, 2000; Kleter and Kuiper, 2002). Two important issues have emerged and have led to investigations worldwide involving multiple GM crops, transgenes, and animal species. The first issue concerns the potential for integration of transgenes into genomes of bacteria found residing in the gastrointestinal tracts of livestock and subsequent expression of the transgenes. The second issue relates to the absorption of transgenic DNA across the intestinal epithelium of livestock and its occurrence in animal products. The objectives of this thesis were to investigate the fate of glyphosate tolerant canola (Roundup Ready®, R) and transgenic DNA throughout feed processing and ruminant digestion, in comparison to a parental, near isogenic line of canola, and endogenous canola DNA. Based on data from those investigations, a subsequent study applying real-time PCR to estimate ruminal dry matter (DM) digestion of feeds in a mixed diet was examined.

Feed processing and in particular, applied heat and grinding, can lead to plant DNA fragmentation (Gawienowski et al., 1999; Chiter et al., 2000; Yoshimura et al., 2005). Therefore, the processing effects on transgenic and endogenous DNA in R and parental canola meals, and diets containing each meal-type, were investigated (Chapter 2). Despite temperatures reaching 100°C during canola meal preparation and processing of the diets, DNA fragments up to 23 kbp remained. Detection of the full length 5-enolpyruvylshikimate-3-phosphate synthase transgene (*cp4 epsps*, 1363 bp) from the CP4 strain of *Agrobacterium tumefaciens* was confirmed in R meal and diet. Additionally, a 544 bp fragment of the *ribulose-1,5-bisphosphate carboxylase/oxygenase* (*Rubisco*) small subunit gene was amplifiable in R and parental meals, and diets. This indicated that relatively large fragments of plant DNA and entire transgenes would be ingested by livestock fed processed GM canola.

In vitro incubations of seeds, meals and diets in ruminal batch cultures revealed that digestion of R canola substrates was similar in comparison to parental controls. Total volatile fatty acid production (Chapter 2) and ammonia concentrations and gas accumulations (Chapter 3) were not significantly different between R and parental canola substrates. Ruminal fermentation was only affected by the extent of feed processing and was greatest for diets followed by meals, cracked seeds, and whole seeds. This reflected the enhanced availability of digestible internal components resulting from processing. Substantial equivalence is a policy used by many regulatory agencies to evaluate the safety of GM crops by comparing similarities and differences

between a GM variety and normally the parental line from which it was derived (FAO/WHO, 2000). From Chapters 2 and 3, it appeared that R canola was digested equivalently compared to parental canola, in terms of fermentation end-products. These results are supported by a previous study testing the digestibility, average daily feed gain, feed efficiency, and carcass characteristics in lambs fed diets containing meal from R and parental canola varieties (Stanford et al., 2003). In that study, Stanford et al., (2003) reported similar responses from the R and parental diets.

In Chapter 2, persistence of a 544-bp fragment of the endogenous *Rubisco* gene and the 1363 bp *cp4 epsps* transgene were studied. Detection of the *Rubisco* gene fragment was inversely related to substrate digestibility (amplifiable for 48, 8 and 4 h in whole or cracked seeds, meal and diets respectively), but did not differ between parental and R canola. Furthermore, the transgene persisted for the same length of time compared to the *Rubisco* fragment in R canola, demonstrating similar degradation of transgenic and endogenous DNA. For every substrate tested, both endogenous and transgenic plant DNA were associated only with solid canola substrate, implying rapid degradation upon release to the aqueous ruminal environment. In support of this finding, previous studies have shown the rapid degradation of free DNA in rumen contents due to microbial nuclease activity (Flint and Thomson, 1990; Duggan, et al., 2000; Ruiz et al., 2000). The instability of free DNA in rumen fluid likely impedes the potential for gene transfer from plants to bacteria, an event that was not detected when analyzing the *cp4 epsps* transgene or *Rubsico* fragment.

High-molecular weight DNA has been shown to have a short half life in rumen fluid (Ruiz et al., 2000). Therefore, in Chapter 3, DNA fragments smaller than those investigated in Chapter 2 were analyzed. Seven fragments of transgenic DNA ranging in size between 179 to 527 bp and spanning the *cp4 epsps* construct were tracked. In addition, a 180-bp fragment of the *Rubisco* gene was used as a control for detecting endogenous canola DNA. Each of the transgene fragments and the *Rubisco* gene were detected for equal lengths of time. As in the case of the 1363-bp *cp4 epsps* transgene and the 544-bp fragment of *Rubisco* studied in Chapter 2, detection of canola DNA was dependent on processing and was possible for up to 48, 8 and 4 h in whole or cracked seeds, meal and diets respectively. None of the smaller fragments of DNA were amplifiable in aqueous rumen fluid, demonstrating uniform degradation throughout the transgene construct and between transgenic and endogenous plant DNA. Phipps et al. (2003), showed that transgenic and endogenous DNA could be detected to similar extents throughout the digestive tract of cattle, so long as the copy number of both genes tested for were present at comparable levels. The *Rubisco* small subunit gene is a low-copy nuclear encoded gene (Beck et al., 1995) and the transgenic construct in R canola is single copy, which likely explains the similar detection

patterns. None of the transgenic fragments were amplifiable in microbial DNA, suggesting that transformation with smaller DNA fragments was no more likely than the entire *cp4 epsps* transgene.

The inability to detect canola DNA in the aqueous supernatants of ruminal fluid implied rapid degradation of free DNA when released from plant material. To test this, free DNA from R canola seed was incubated in rumen fluid and transgenic DNA detection was assessed (Chapter 4). Fragments between 300 to 1363 bp in size were detected up to 10 min. Together, this finding and the data from Chapters 2 and 3, suggested that survival of free transgenic DNA released from plant cells in the rumen would unlikely survive passage to the small intestine. Therefore, free transgenic DNA available for absorption across the intestinal epithelium would have to be released somewhere distal to the abomasum. The potential for digestion of R canola and release of transgenic DNA in the small intestine was analyzed by real-time PCR in duodenal batch culture incubations. At neutral pH, similar to that of the ileum, transgene copy number declined rapidly between 8 and 16 h of incubation, after an initial 4-h lag. Disappearance of the transgene was inversely related to microbial digestion, as measured by total volatile fatty acid production. The 62-bp fragment of transgenic DNA detected by real-time PCR reached a maximum of approximately 1600 copies in the aqueous phase of duodenal fluid. However, the 1363-bp *cp4 epsps* transgene was never detected in the aqueous fraction. Free DNA, which would be released upon plant cell digestion, persisted for 2 min in duodenal fluid at pH 7, when the fragment size ranged between 300 to 527 bp. Detection was only possible for up to 0.5 min when the fragment analyzed was 1363 bp. This study showed that digestion of plant material and release of transgenic DNA can occur in the ruminant small intestine. However, free DNA is rapidly degraded at neutral pH in duodenal fluid, thus reducing the likelihood that intact transgenic DNA would be available for absorption in the distal ileum.

The findings from *in vitro* investigations in Chapters 2, 3, and 4, were substantiated in an *in vivo* study (Chapter 5). The fate of transgenic DNA was investigated using six ruminally and duodenally canulated wethers (Chapter 5) fed either forage- or concentrate- based diets containing 15% (w/w; as fed) R canola meal. A 108-bp fragment of transgenic DNA was quantifiable for up to 21 h in whole ruminal and duodenal fluid after the animals were taken off GM diets. The quantity of transgenic DNA in ruminal and duodenal fluids was not different between forage- and concentrate- based diets, indicating that fibre content did not affect transgene persistence. However the quantity of transgenic DNA was significantly greater in ruminal fluid compared to duodenal fluid, which was likely due to degradation of canola DNA in the rumen. Although transgenic DNA reached the small intestine, it could not be detected in fecal material,

suggesting extensive digestion distal to the duodenum. Fragments of transgenic DNA could not be detected in aqueous ruminal fluid, similar to ruminal batch culture incubations in Chapters 2 and 3. Additionally, it was not observed in aqueous duodenal fluid, reinforcing the notion that for free transgenic DNA to occur in the small intestine, it must be released there. This is supported by Phipps et al. (2003), who were unable to detect low copy plant genes in duodenal fluid of cattle.

Transfer of transgenic DNA to microflora of the rumen and duodenum was not identified *in vivo* and seems to be limited by extensive degradation of free DNA like it is *in vitro*. Similarly, plant DNA instability in digesta contents impedes absorption across the intestinal barrier. Despite this, fragments of transgenes have been found in meat and milk from cattle (Nemeth et al., 2004). Smaller fragments of DNA appear to be absorbed to a greater extent that larger fragments (Nemeth et al., 2004). Therefore, blood DNA from wethers, while being fed GM diets, were analyzed for fragments of transgenic DNA ranging in size from 62 to 420 bp. None of the fragments were detected. To date, several studies have identified high-copy plant genes in livestock products (Einsapnier et al., 2001; Klotz et al., 2003; Reuter and Aulrich, 2003; Tony et al., 2003; Nemeth et al., 2004; Aeschbacher et al., 2005; Mazza et al., 2005;), but only two have reported positive results for transgenic DNA (Nemeth et al., 2004; Mazza et al., 2005). Absorption of transgenic DNA has not been reported to occur at levels greater than endogenous plant DNA (Nemeth et al., 2004; Mazza et al., 2005). Such an event is dependent on the amount of transgenic DNA reaching the small intestine. In Chapter 5, GM diets contained 15% (w/w; as fed) R canola meal, most of which was digested in the rumen. The DNA from R canola meal, at most, only accounted for 0.18% (w/w) of the total DNA in duodenal contents. Such a dilution likely restricted absorption and the ability to detect transgenic fragments in blood.

From Chapters 2, 3, 4, and 5, it was evident that low copy plant genes within the digestive contents of ruminants were limited to solid plant substrate. In Chapter 4, decrease in transgene copy number strongly correlated to volatile fatty acid production (r = -0.98) throughout incubation in duodenal batch cultures. These findings led to the hypothesis that plant DNA disappearance, quantified by real-time PCR, could be used to estimate dry matter (DM) disappearance of individual feeds in a mixed diet (Chapter 6). To test the hypothesis, regression analysis between DNA and DM disappearance for 100% alfalfa or corn, throughout ruminal incubations at pH 6.8 or 5.5, was used to predict DM disappearance of single feeds in mixed diets containing 75:25, 50:50, and 25:75 ratios of alfalfa to corn. This method to predict DM disappearance was relatively accurate, having a concordance correlation of 0.87. Alfalfa DM disappearance was found to be influenced by pH for all mixed diets and the microflora

responsible for digesting corn when corn was present at its highest concentration. Both associative effects have previously been suggested to affect forage DM disappearance (Mould et al.,1983; Hoover, 1986). Corn digestion was only affected by time. Although further research is warranted, for the first time, a direct method to predict the DM disappearance of individual feed components in a mixed diet, while considering associative effects, has been presented.

In summary, the fate of plant DNA fragments has been investigated throughout the ruminant digestive tract. Transgenic plant DNA has received interest since the commercialisation of GM crops. Both FAO and WHO have stated that transgenic DNA is no more likely to pose a health risk than endogenous DNA because all DNA is of similar structure and degraded in a similar fashion (FAO/WHO, 1991). In all respects studied (e.g., degradation during feed processing, fermentation characteristics, stability of DNA upon exposure to deoxyribonucleases in digestive fluids) the transgenic DNA encoded in R canola was indistinguishable from endogenous DNA. Together, microbial digestion of R canola substrate, the highly unstable nature of free transgenic DNA in ruminal and intestinal digesta, and the dilution of transgenic DNA in endogenous plant DNA and microbial DNA all reduced the potential for transfer of even small transgene fragments to microbes within the digestive tract of sheep and across the intestinal barrier. The association of low copy plant genes to only solid plant material in digesta contents gave rise to a novel method of estimating DM disappearance of individual feeds in a mixed diet, by utilizing real-time PCR. This method of predicting DM digestibility may and lead to redefined models to more effectively predict the available energy of total mixed diets. Ultimately, economic benefits from new models to formulate mixed diets may occur.

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